METHOD DEVELOPMENT AND VALIDATION OF PARACETMOL AND FLUPIRTINE MALEATE BY RP-HPLC

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LIST OF ABBREVIATIONS USED

HPLC	-	High Performance Lliquid Cchromatography
HILIC	-	Hydrophilic Interaction Chromatography
UV	-	Ultra Violet
S.D	-	Standard Deviation
%RSD	-	Relative Standard Deviation
R_t	-	Retention Time
RP-HPLC	-	Reverse Phase High Performance Liquid Chromatography
NP- HPLC	-	Normal Phase High Performance Liquid Chromato graphy
RPC	-	Reversed Phase Choromatography
SEC	-	Size Exclusion Chromato graphy
ANP	-	Aqueous Normal Phase Chromatography
ONP	-	Organic Normal Phase Chromatography
P^H	-	Negative Logarithm of Hydrogen Ion
Ml	-	Milliliter
Mg	-	Milligram
LOQ	-	Limit Of Quantification
LOD	-	Limit Of Detection
ppm	-	Parts per million
CZE	-	Capillry Zone Electrophoresis
PC	-	Paracetmol
FM	-	Flupirtine maleate

1. INTRODUCTION

Analytical methods development and validation play important roles in the discovery, development, and manufacture of pharmaceuticals. The current good manufacturing practice (CGMP) and Food Drug Administration (FDA) Guidelines insist for adoption of sound methods of analysis with greater sensitivity and reproducibility. Development of a method of analysis is usually based on prior art (or) existing literature, using the same (or) quite similar instrumentation .It is rare today that an HPLC-based method is developed that does not in same way relate (or) compare to existing, literature based approaches. Today HPLC (High performance liquid chromatography) is the method of choice used by the pharmaceutical industry to assay the intact drug and degradation products. The appropriate selection and chromatographic conditions ensure that the HPLC method will have the desired specificity. UV spectroscopy is also a simple analytical tool widely used for routine assay of drugs. Hence for the assay of the selected drugs HPLC and UV spectroscopy has been chosen for these proposed methods[11].

The developed chromatographic methods further validated as per ICH or USFDA guidelines for all the critical parameters. To access the precision and to evaluate the results of analysis the analyst must use statistical methods. These methods include confidence limit, regression analysis to establish calibration curves. In each analysis the critical response parameters must be optimized and recognized if possible.

Pharmaceutical Analysis plays a major role today, and it can be considered as an interdisciplinary subject. Pharmaceutical analysis derives its principles from various branches like chemistry, physics. And microbiology etc. pharmaceutical

Analytical techniques are applied mainly in two areas, quantitative analysis and qualitative analysis, although there are several other applications.

Drugs and pharmaceuticals are chemicals or like substances, which or of organic inorganic or other origin. Whatever may be the origin, we some property of the medicinal agent to measure them quantitatively or qualitatively.

In recent years, several analytical techniques have been evolved that combine two or more methods into one called "hyphenated" technique eg: GC/MS, LC/MS etc. The complete Analysis of a substance consists of four main steps.

The concept of analytical chemistry lies in the simple, precise and accurate measurements. These determinations require highly sophisticated instruments and methods like mass spectroscopy, gas chromatography, HPTLC, HPLC, etc. HPLC method is sensitive, accurate, precise and desirable for routine estimation of drugs in formulations.

Thereby it is advantageous than volumetric methods. Many HPLC methods has been developed and validated for the quantitative determination of various marketed drugs.

Analytical method development and validation places an important role in drug discovery and manufacture of pharmaceuticals. These methods are used to ensure the identity, purity, potency and performance of drug products majority of analytical development effort goes into validating a stability indicating method. So it is a quantitative analytical method based on the structure and chemical properties of each active ingredient of the drug formulation.

Most of the drugs can be analyzed by HPLC method because of several advantages like rapidity, specificity, accuracy, precision, reproducibility, ease of automation and eliminates tedious extraction and isolation procedures.

On the literature survey, it was found that most of the analytical method available for the above mentioned drug is applicable for quantification in plasma samples, the most widely used method being liquid chromatography-mass chromatography. So it is felt that there is a need to develop accurate, precise analytical methods for the estimation of the drug in solid dosage formulation[1].

Newer analytical methods are developed for these drugs or drug combinations of the below reasons:

- There may not be suitable method for a particular analyte in the specific matrix.
- Existing method may be too error prone or unreliable (have poor accuracy and precision).
- Existing method may be expensive, time consuming, energy intensive and may not be provide sensitive or analyte selectivity, and not easy for automation.
- Newer instrumentation and techniques may have evolved that provide opportunities for improved methods.
- There may be need for an alternate method to confirm, for legal and scientific reasons.

The newly developed analytical methods having their importance in different fields that include, research and development centre (R&D), Quality control

department (QC). Approved testing laboratories, chemical Analysis laboratories etc. For analysis of these drugs different analytical methods are routinely being used[4].

The analytical methods are classified as classical and instrumental. These methods signal measured in those methods was mentioned in following table.

Table 1 : Classification of analytical method

Measurement signal	**Analytical method**
Chromatographic techniques	
Electrical	Gas chromatography (Thermal conductivity detector)
Increase in electrical current	Gas chromatography (Flame ionization detector)
Decrease in electrical current	Gas chromatography (Flame capture detector)
Electromagnetic radiation absorbed	Liquid chromatography (Ultraviolet Light detector, Diode array detector)
Electrical	Ion chromatography
Spectrophotometric method	
Emission radiation	Emission spectroscopy (X-ray, UV, Visible), Fluorescence and phosphorescence (X-ray, UV, Visible),radiochemistry.
Absorption of radiation	Spectrophotometry (X-ray, UV, Visible, IR) NMR and Electron spin resonance spectroscopy.
Scattering of radiation	Turbidimetry, Nephelometry, Raman spectroscopy
Refraction of radiation	Refractometry, Interferometry
Diffraction of Light	X-ray and Electron diffraction
Rotation of radiation	Polarimetry, Optical rotatory dispersion
Mass to charge ratio	Mass spectroscopy
Electro chemical techniques	
Electrical potential	Potentiometry
Electrical current	Polarography, Amperometry
Electrical resistance	Conductometry
Miscellaneous techniques	
Rate of reaction	Kinetic method
Thermal properties	DTA.DSC
Classical methods	
Mass	Gravimetric Analysis
Volume	Volumetric Analysis[2]

2. CHROMATOGRAPHY

Techniques related to chromatography have been used for centuries to separate materials such as dyes extracted from plants. Russian botanist Tswett is credited with the discovery of chromatography. In 1903 he succeeded in separating leaf pigments using a solid polar stationary phase, It was not until 1930s that this technique was followed by Kuhn and Lederer as well as Reichstein and van Euw for the separation of natural products. Martin and synge were awarded the Nobile prize for their work in 1941 in which they described liquid-liquid chromatography. Martin and synge applied the concept of theoretical plates as a measure of chromatographic efficiency. The term "chromatography" (Color-writing derived from the Greek for Color-chroma and Write-Graphing)[18].

CHROMATOGRAPHY IN THE PHARMACEUTICAL WORLD

In the modern pharmaceutical industry, chromatography is the major and integral analytical tool applied in all stages of drug discovery, development, and production. The development of new chemical entities (NCEs) is comprised of two major activities. Drug discovery and development. The goal of the drug discovered is to investigate a plethora of compounds employing fast screening approaches, leading to generation of lead compounds and then narrowing the selection through targeted synthesis and selective screening (lead optimization). The main functions of drug development are to completely characterize candidate compounds by performing drug metabolism, preclinical and clinical screening, and clinical trails. Throughout this drug discovery and development paradigm, rugged analytical HPLC separation methods are developed, at each phase of development to analyses of a myriad of samples are performed to adequately control and monitor the quality of the

prospective drug candidates, excipients, and final products. Effective and fast method development is of paramount importance throughout. This drug development life cycle. This requires a thorough understanding of HPLC principles and theory which have solid foundation for appreciating the many variables that are optimized during fast and effective HPLC method development and optimization[3].

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

3.1 Brief Historical prospective of chromatography:

The historical development of liquid chromatography has been extensively reviewed and can be traced as far back as they early 1900, where the Russian botanist Zwett used a variant of liquid chromatography to separate some colored plant substances. The focus was on modern development in HPLC, a term that was coined in late 1960s with the advent of more sophisticated instrumentation, better engineered separation columns, and reliable and highly efficient stationary phases and packaging materials.

These technological advances have been, In part, fuelled, by the need to separate an increasingly large variety of differing compounds classes encountered as API s, e.g. Antibiotic, sulphonamides nucleosides, fat soluble vitamins neutral and non polar compounds. Additional challenges include developing faster and more consistent HPLC methods requiring higher flow rates, while maintaining peak shape, peak symmetry and efficiencies. Another important analytical challenge is the desire to detect and accurately quantify low levels of impurities at level present in API materials.

High-pressure liquid chromatography quickly improved with the development of column packing materials. Additional convenience of on-line detectors became rapidly a powerful separation technique and is today called as High-performance liquid chromatography (HPLC)

- One of the early problems with liquid state chromatography was the slow rate at which analysis took place. Early methods use gravity feed, and it was not uncommon diffusion and soon.

- This problem was largely overcome by the advent High-performance liquid Chromatography (HPLC). In this system the pressure is applied to the column forcing the mobile phase through at much higher rate.

- For an analysis to take several days to complete. This led not only to great delay but also the excessive time on the column and thus inevitably led to loss of resolution by

Table: 2. Different types of chromatographic techniques

S. No.	Basic principle involved	Type of chromatography
1	Techniques by chromatographic bed shape	Column chromatography
		Paper chromatography
		Thin layer chromatography
2	Techniques by physical state of mobile phase	Gas chromatography
		Liquid chromatography
3	Affinity chromatography	Super critical fluid chromatography
4	Techniques by separation mechanism	Ion Exchange chromatography
		Size Exclusion chromatography
5	Special techniques	Reversed phase chromatography
		Two-dimensional chromatography

HPLC

In High-performance liquid chromatography, mobile as well as the stationary phase compete for the distribution of the sample components. In case of HPLC, separation is based on adsorption and partition. Adsorption chromatography employs high-surface area particles that adsorb the solute molecules. Usually a polar solid such as silica gel, alumina or porous glass beads and a non-polar mobile phase such as heptanes, octane or chloroform are used in adsorption chromatography.

In partition chromatography, the solid support is coated with a liquid stationary phase. The relative distribution of solutes between the two liquid phases determines the separation. The stationary phase can either polar or non-polar. If the stationary phase is non-polar, it is called normal phase partition chromatography. If the opposite case holds, it is called reversed-phase partition chromatography. In normal phase mode, the polar molecule partition preferentially in to the stationary phase and are retained longer than non-polar compounds. In reverse phase partition chromatography, the opposite behavior is observed[8].

3.2 TYPES OF HPLC TECHNIQUES:

Based on modes of chromatography:

- Normal phase chromatography
- Reverse phase chromatography

Based on principle of separation:

- ❖ Adsorption chromatography
- ❖ Ion exchange chromatography

- Size exclusion chromatography
- Affinity chromatography

Based on elution technique:

- Isocratic separation
- Gradient separation

Based on the scale of operation:

- Analytical HPLC
- Preparative HPLC

Ion Exchange chromatography: Due to differences in the affinity of ions for the in exchange.

Size Exclusion chromatography: Due to differences in molecular weight and size of the molecules to be separated.

Affinity chromatography: Separation is based on a chemical interaction specific tothe target species. The more popular revered phase mode uses a buffer and an added counter-ion of opposite charge to the sample with separation being influenced by pH, ionic strength, temperature, concentration of and type of organic co-solvents(s).

Chiral chromatography: Separation of the enantiomers can be achieved on chiral stationary phases by the formation of diastereomers.

Analytical HPLC: only analysis of the samples is done. Recovery of the samples for reusing is normally not done[6].

3.3 MOST COMMONLY USED METHODS IN HPLC

Normal phase chromatography:

For a polar stationary bed like silica we need to choose a relatively non-polar Mobile phase. This mode of operation is termed as Normal phase chromatography. Here the least polar component elutes first, and increasing the mobile phase polarity leads to decrease in elution time. Non-polar solvents like pentane, Hexane, isooctane, cyclohexane, etc. are more popular. It is mainly used for separation of nonionic, non-polar to medium polar substances.

Reverse phase chromatography:

In 1960s, chromatographers started modifying the polar nature of the silanol group by chemically reacting silicon with organic silanes. The object was to make silica less polar or non-polar so that polar solvents can be used to separate water-soluble polar compounds. Since the ionic nature of the reverted, the chromatographic separation carried out with such silica is referred to as Reverse- phase chromatography. Here the most post components elutes first. Increasing mobile phase polarity leads to decrease In elution time. common solvents used in this mode include Methanol /Acetonitrile /Isopropanal etc. Mostly used for separation of ionic and polar substances. The parameters that govern the retention in reversed phase system are the following:

a. The chemical nature of the stationary phase surface.

b. The type of solvents that compose the mobile phase.

c. pH and ionic strength of the mobile phase.

Isocratic elution: A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition).

Gradient elution: The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a gradient elution[10].

3.4 INSTRUMENTATION OF HPLC:

The mobile phase components HPLC instrument and their working functions are described below.

- Mobile phase and reservoir
- Solvent degassing system
- Pump
- Injector
- Colum
- Detector
- Data system

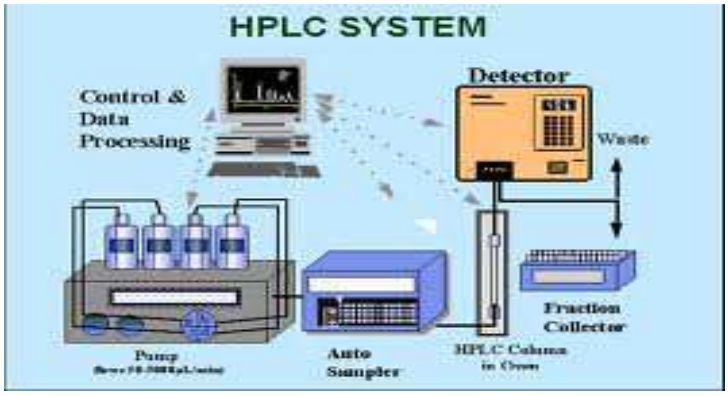


Figure1: Schematic diagram of HPLC instrumentation

I.MOBILE PHASE AND RESERVIOR:

The most common type of solvent reservoir is a glass bottle. The mobile phase is pumped under pressure from one of several reservoirs and flows through the column at a constant rate. With micro particulate packing, there is a high-pressure drop across a chromatography column. Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous buffers. The following points should also be considered when choosing amobile phase:

- The essential to establish that the drug is stable in the mobile phase for at least the duration of the analysis.
- Excessive salt concentrations should be avoided. High salt concentrations can result inprecipitation, which can damage HPLC equipment.
- The mobile phase should have a pH 2.5 and Ph 7.0 to maximize the lifetime of the column.
- Reduce cost and toxicity of the mobile phase by using methanol instead of acetonitrile when possible minimizes the absorbance of buffer.
- Use volatile mobile phase when possible, to facilitate collection of products and LC-MS analysis. Volatile mobile phases includes ammonium, acetate, ammonium phosphate, formic acid, and trifluoroacetic acid. Some caution is needed as these buffers absorb below 220nm.

Mobile phase used for HPLC are typically mixtures of organic solvents and water or aqueous Buffers. Physical properties of some HPLC solvents were summarized in table:3

Table: 3. Physical properties of common HPLC solvents

Solvent	MW	BP	RI ($25^{O}C$)	UV λ Cut-off(nm)	Density g/ml($25^{O}c$)	Viscosity C_P($25^{O}C$)	Dielectic constant
Acetonitrile	41.0	82	1.342	190	0.787	0.358	38.8
Dioxane	88.1	101	1.420	215	1.034	1.26	2.21
Ethanol	46.1	78	1.359	205	0.789	1.19	24.5
Ethyl Acetate	88.1	77	1.372	256	0.901	0.450	6.02
Methanol	32.0	65	1.326	205	0.792	0.584	32.7
CH_2CI_2	84.9	40	1.424	233	1.326	0.44	8.93
Isopropanol	60.1	82	1.375	205	0.785	2.39	19.9
N-propanol	60.1	97	1.383	205	0.804	2.20	20.3
THF	72.1	66	1.404	210	0.889	0.51	7.58
a :The wavelength at which the absorbance of 1cm is 1.0							

II. SOLVENT DEGASSING SYSTEM

The constituents of the mobile phase should be degassed and filtered before use. Several methods are employed to remove the dissolved gases in the mobile phase. They include heating and stirring, vacuum degassing with an aspirator, filtration through 0.45µfilters, vacuum degassing with an air-soluble membrane, helium purging ultra signification or purging or combination of these methods. HPLC systems are also provided an online degassing system, which continuously removes the dissolved gases from the mobile phase.

III. PUMP:

High pressure pumps are needed to force solvents through packed stationary phase beds. Smaller bed particles requires higher pressures. There are many advantages to using smaller particles, but they may not be essential for all separations.

The degree of flow of control also varies with pump expense. More expensive pumps include such state of the art technology as electronic feedback and multithreaded configurations. It is desirable to have an integrated degassing system, either helium purging, or membrane filtering.

IV. INJECTOR:

Sample introduction can be accomplished in various ways. The simplest method. Is touse an injection value in more sophisticated LC systems, automatic sampling devices are incorporated where the sample is introduced with the help of auto samplers and microprocessors in liquid chromatography, liquid samples may be injected directly and solid samples need only be dissolved in an appropriate solvent. Sample introduction techniques can be used with a syringe an injection valve.

V. COLUMN:

The heart of the system in the column. Many different reverse phase columns will provide excellent specificity for any particular separation. It is therefore best to routinely attempt separations with a standard C8 or C18 column and determine if it provides good separations. Reverse phase columns differ by the carbon chain length, degree of end capping and percent carbon loading. Diol, cyano and amino groups can also be used for reverse phase chromatography. Typical HPLC columns are 5,10,15, and 25cm in length and are filled with small diameter (3,5 or 10µm) particles. The

internal diameter of the columns is usually 4.6mm; this is considered the best compromise for sample capacity, mobile phase consumption, speed and resolution. However, if pure substances are to be collected (preparative scale),then larger diameter columns may be needed.

VI. DETECTOR:

The detection of UV light absorbance offers both convenience and sensitivity for molecules. when a chromophore is present, the wavelength of detection for a drug should be based on its UV spectrum in the mobile phase and not in pure solvents. The most selective wavelength for detecting a drug is frequently the longest wavelength maximum to avoid interference from solvents, Buffers and Excipient. Other method of detection can be useful are required in some instances.

1. Solute specific detectors (UV-Vis, Fluorescence, Electrochemical, Infra-red, Radio activity)
2. Bulk property detectors (Refractive index, Viscometer, conductivity)
3. Desolvation detector (Flame ionization etc)
4. LC-MS detectors
5. Reaction detectors

VII. DATA SYSTEM:

Since the detector signal is electronic, using modern data collection techniques can aid the signal analysis. In addition, some systems can store data in a form for highly sophisticated computer analysis at a later time. The main goal in using electronic data systems is to increase analysis accuracy and precision, while reducing operator attention.

PERFORMANCE CALCULATIONS:

Calculating the following values (which can be included in a custom report) used to access overall system performance.

1. Relative retention
2. Theoretical plates
3. Capacity factor
4. Resolution
5. Peak asymmetry
6. Plates per meter

The following information furnishes the parameters used to calculate these system performance values for the separation of two chromatographic components. (Note: where the terms w and t both appear in the same equation they must be expressed the same units)

System suitability parameters:

The theory of chromatography has been used as the basis for system-suitability tests, which are set of quantitative criteria that test the suitability of the chromatographic system to identify and quantify drug related samples by HPLC at any step of the pharmaceutical analysis.

1. **Relative retention:** The time elapsed between the injection of the sample components in to the column and their detection is known as the retention time (Rt).

$$\alpha = (t_2 - t_a) / (t_1 - t_a)$$

Where,

α =Relative retention

t_1 = Retention time of the peak measured from point of injection.

t_2 = Retention time of the second measured from point of injection.

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

2. Theoretical plates:

$$n = 16 (t_R / w)^2$$

Where,

n =Theoretical plates

t_R = Retention time of the component

W = width of the base of the component peak using tangent method.

3. Capacity factor: The capacity factor describes the thermodynamic basis of the separation and its definition is the ratio of the amounts of the solute at the stationary and mobile phases within the analyte band inside the chromatographic column.

$$K^1 = (t_2/t_a)-1$$

Where,

K^1 = Capacity factor

t_a = Retention time of an inert peak not retained by the column, measured from point of injection.

4. Resolution: the gap between two peaks

$$R = 2 (t_2 . t_1) / (w_2 - w_1)$$

Where,

R =Resolution between a peak of interest (peak 2) and the peak preceding it (peak 1)

W_2 = Width of the base of component peak 2

W_1 = Width of the base of component peak

5. Peak asymmetry

$$T = W_{0.05} / 2f$$

Where,

T = Peak asymmetry, or tailing factor.

$W_{0.05}$ = Distance from the leading edge to the tailing edge of the peak,measured at a point 5 % of the peak height from the baseline.

f= Distance from the peak maximum to the leading edge of the peak.

6. PLATE PER METER:

$$N = n/L$$

Where,

N = plates per meter.

L = column length in meters.

Advantages:

- HPLC separations can be accomplished in a minutes, in some cases even in seconds.

- High resolution of complex sample mixture into individual components.
- Rapid growth of HPLC is also because of its ability to analyse substances that are unsuitable for Gas Liquid chromatographic (GLC) analysis due to non-volatility orthermal-instability.
- Quantitative analysis are easily and accurately performed and errors of less than 1 % are common to most HPLC methods.
- Depending on sample type and detector used, it is frequently possible to measure 10^{-9} g or 1 ng of sample. With special detectors, analysis down to 10-12 pg has been reported.
- As HPLC is versatile, it can be applied to wide variety of samples like organic, inorganic, high molecular weight liquids, solids, ionic-nonionic compounds.

Disadvantages:

- HPLC instrumentation is expensive and represents a major investment for many laboratories.
- HPLC cannot handle gas samples.
- HPLC is poor identifier. It provides superior resolution but it does not provide the information that identifies each peak.
- Only one sample can be analysed at a time.

  Finally, at present there is no universal and sensitive detector.

4. ANALYTICAL METHOD DEVELOPMENT

Methods are developed for new products when no official methods are available. Alternate methods for existing (non-pharmacopoeial) products are

developed to reduce the cost and time for better precision and ruggedness. Trail runs are conducted, method is optimized and validated.

When alternate method proposed is intended to replace the existing procedure, comparative laboratory data includes merits /demerits should be made available.

The important factors, which to be taken into account to obtain reliable quantitative analysis , are

1. Careful sample and sample preparation
2. Appropriate choice of the column
3. Selection flow rate
4. Selection of detector wavelength
5. Selection of column temperature

Documentation starts at the very beginning of the development process. A system for full documentation of development studies must be established. Analyte standard characterization.

a) All known information about the analyte and its structure is collected i.e., physical and chemical properties.

b) The literature for all type of information related to the analyte is surveyed.

c) Using the information in the literatures and prints, methodology is adapted. The methods are modified where ever necessary.

d) The required instrumentation is setup. Installation, operational and performance qualification of instrumentation using laboratory SOP's are verified[9].

HPLC method development is based on few basic steps which include:

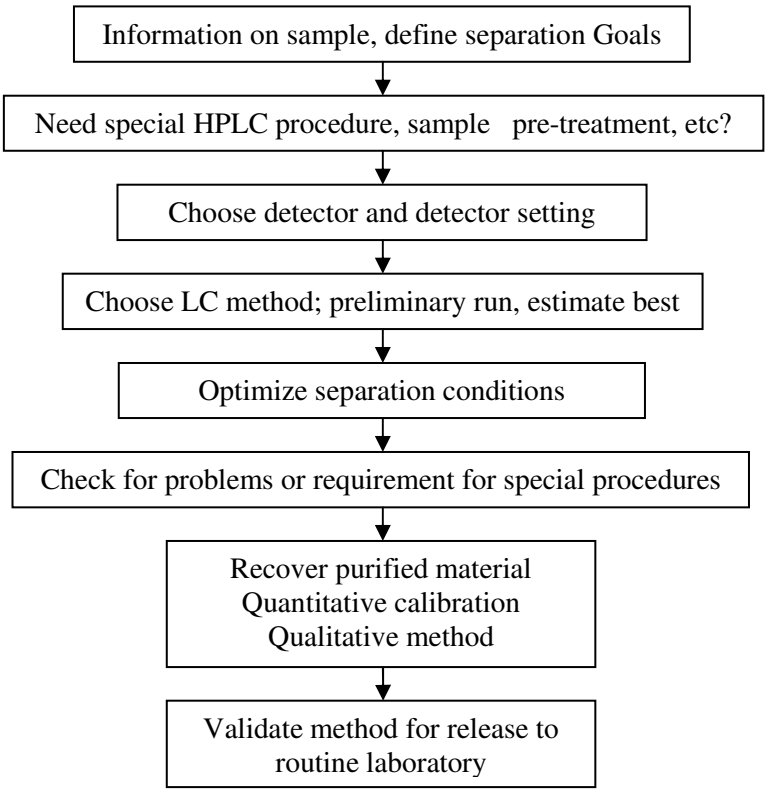


Figure 2. Steps in HPLC method development

Method goals:

Analytical method goals are often defined as method acceptance criteria for peak resolution, precision, specificity, sensitivity. For instance, pharmaceutical methods for potency assays of an API typically require the following:

- Minimal sample work-up (extra and inject if possible
- Robust method that doesn't require extensive execution.
- Low cost per analysis.

Table 4: separation goals in HPLC method development

Goals	Comment
Resolution	Precise and rugged quantitative analysis requires that Rs be greater than 1.5.
%RSD	Precision of retention time and peak area, ‹1-2%RSD.
Range	Linearity in the range of 50-150% of the lab label claim.
Analysis time	□5-30min (□60min for complex samples)
Separation time	‹5-10min is desirable for routine procedures.
Quantification	≤2%(%RSD) for assays, ≤5% for less demanding analyses, ≤15% for trace analyses.
pressure	‹150 bar is desirable, ‹200bar is usually essential(new column assumed)
Peak height	Narrow peaks are desirable for large signal/ noise ratios

Sample analyte information:

The information is useful for the selection of appropriate sample preparation procedures as well as the initial detection and chromatographic modes. If data not available (e.g., PKa solubility), separate studies should be initiated as soon as possible. The sample related information is summarized in Table 4.

Table 5: sample and analyte information

Sample/analyte	Information
Sample	Number of components concentration range of analytes
Analyte (s)	Chemical structure, molecular weight and functional groups PKa, Solubility, Chrpmophores, wavelength (max), Chiral centers, isomers Spectral data (MS,NMR, IR, and UV) Stability and toxicity
Others	Purity of reference standard materials

1. Careful sampling and sample preparation:

Before beginning method development, it is need review what is known about the sample in to define the goals of separation. The sample related information that is important to summarized in table. The chemical composition of the sample can be provide valuable clues for the best choice of initial conditions for an HPLC separation.

- Number of compounds present
- Molecular weight of compounds
- PKa values of compounds
- UV spectra of compounds
- Concentration range of compounds in samples of interest
- Sample solubility

2. Separation goals

The goals of HPLC separation need to be specified clearly, which include

- The use of HPLC to isolate purified sample components for spectral identification Or quantitative analysis
- It may necessary to separate all degradants or impurities from a product for reliable content assay or not
- In quantitative analysis, the required levels of accuracy and precision should be known

- Whether a single HPLC procedure is sufficient for raw materials or one or more different procedures are desired for formulations

- When the number of samples for analysis at one time is greater than 10, a run time of less than 20 minutes often will be important. Knowledge on the desired HPLC equipment.

- HPLC equipment, HPLC experience and academic training do to operators have

Sample preparation: samples come in various forms

- Solutions ready for injection

- solutions that require dilution, buffering, addition of an internal standard or other volumetric manipulation

- solids must be dissolved or extracted

- Samples that require pretreatment to remove interference and / or to protect the column or equipment from damage

3. Appropriate choice of the column:

The selection of the column in HPLC is somewhat similar to the selection of column in G.C, in the sense that, in the adsorption and partition modes, the separation mechanism is based on inductive forces, dipole-dipole interaction and hydrogen bond information.

Column plays the important role in achieving the chromatographic separation. The following parameters should be considered while selecting a column

i. Length and diameter of the column

ii. Packing material

iii. Size and shape of the particles

iv. Pore size, surface area and end capping

v. Percentage of carbon loading columns with silica as a packing material used widely in normal phase chromatography, where the eluent (mobile phase) is non-polar consisting of various organic solvents and the stationary phase is polar. The silanol groups on the surface of the silica give it a polar character.

In reverse phase chromatography a wide variety of columns is available covering a wide range of polarity by cross linking the silanol groups with alkyl chains like $_{C6,\ C8,\ C18}$ and Nitrile groups (-CN), phenyl groups ($-_{C6H6}$) and amino groups ($-NH_2$)

ORDER OF THE SILICA BASED COLUMNS

I------Non polar------Moderately polar------Polar------I

$C_{18}<C_8<C_6$‹ Phenyl ‹ Amino ‹ Cyano ‹ Silica

4. Selection of flow rate.

Flow rate is selected based on the follows

- Retention time
- Column composition
- Separation impurities
- Peak symmetry

Preferably flow rate shall not be more than 2.5 mL/Min. a flow rate that gives least retention times, good peak symmetries, least back pressure and better separatiotn of impurities from API peak shell be selected.

5. Selection of detector wavelength:

Selection of detector wavelength is a critical step in finalization of the analytical method. To determine the exact wavelength standard API is injected into chromatographic system with photo Diode array detector and the wavelength, which gives higher response for the Compound.

6. Selection of column temperature:

Ambient temperature is always preferred as a column temperature. However if the peak Symmetry could not be achieved then the column temperature can be varied between 30^0To $_{80}0$ c. if a column temperature above $_{80}0$c is found necessary, packing material which can Withstand to that temperature shall be chosen. The increase in column temperature generally will result in reduction in peak asymmetry and peak retentions.

5. ANALYTICAL METHOD VALIDATION:

Method validation can be defined as (ICH) "Establishing documented evidence, which Provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting is predetermined specifications and quality characteristics".

Method validation study include system suitability, linearity, precision, accuracy, specificity, ruggedness, robustness, limit of detection, limit of quantification and stability of samples, reagents, instruments[13].

VALIDATION DEFINITION:

FDA defines validation as "Establishing documented evidence, which Provides a high degree of degree of assurance that a specific process will consistently produce a product of predetermined specifications and quality attributes.

OBJECTIVE OF METHOD VALIDATION:

The objective of validation is to form a basis for written procedure for production and control, which are designed to assure that the drug products have the identity, quality, and purity.

TYPES OF ANALYTICAL PROCEDURES:

i. Identification tests

ii. Quantitative tests for impurities content

iii. Limit test for control of impurities

iv. Quantitative tests of the active moiety in samples of drug substances or drug product or

v. Other selected components(s) in the drug product.

vi. Dissolution testing for drug products

vii. Particle size determination for drug substances.

6. VALIDATION PARAMETERS (ICH).

Typical validation study include system suitability

I. Accuracy

II. Precision

III. Specificity

IV. Linearity

V. Detection limit

VI. Quantification limit

VII. Range

VIII. Robustnes

1. System suitability

Prior to the analysis of samples of each day, the operator that the HPLC system and proceduce are capable of providing data of acceptable quality. This is accomplished with system suitability experiments, which can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation has been completed.

Table : 6. System suitability parameters and Recommendation

Parameter	Recommendation
Capacity factor	The peak should be well-resolved from Other peaks and the void volume, Generally K› 2.0
Repeatability	RSD ≤1% N ≥ 5 is desirable
Relative retention	Not essential as long as the resolution is Stated
Resolution	RS of › 2 between the peak interest and The closes to eluting potential Interferent (impurity, excipient, degradation product, internal standard, etc,)
Tailing factor	T of ≤ 2
Theoretical plates	N› 2000

Non-Interference of placebo:

The portion of specificity evaluation applies to the finished drug product only. Excipients present in the formulation should be evaluated and must not interfere with the detection of the analyte.

2. Linearity

The linearity of a method is a measure of how well a calibration plot of response vs. concentration approximates a straight line. Linearity can be assessed by performing Single measurement at several analyte concentrations. The data is then processed using a linear least- squares regression. The resulting plot slope, intercept and correlation coefficient Provide the desired information on linearity.

3. Precision

Precision can be defined as "The degree of agreement among individual test results when The procedure is applied repeatedly to multiple samplings of a homogenous sample". A More comprehensive definition proposed by the international conference on Harmonization (ICH) divides precision into three types.

1. Repeatability

2. Intermediate precision and

3. Reproducibility

Repeatability: is the precision of a method under the same operating conditions over a short period of time.

Intermediate precision: is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory.

Reproducibility: examine the precision between laboratories and is often determined in collaborative studies or method transfer experiments.

4. Accuracy:

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, accuracy, is represented and determined by recovery studies. There are three ways to determine accuracy:

1. Comparison to a reference standard
2. Recovery of the analyte spiked into black matrix or
3. Standard addition of the analyte.

It should be clear how the individual or total impurities are to be determined. e.g., weight/ weight or area percent in all cases with respect to the major analyte.

5. Specificity/ selectivity

The terms specificity are often used interchangeably. According to ICH, the term specific generally refers to a method that produces a response for a single analyte only while the term selective refers to a method which provides responses for a number of chemical entities that may or may not be distinguished from each other. If the response is distinguished from all other responses, the method said to be selected. Since there are very few methods that respond to only one analyte, the term selectivity

is more appropriate. The analyte should have no interference from other extraneous components and be well resolved from them. A representative chromatogram or profile should be generated and submitted to show that the extraneous peak either by addition of known compounds or samples from stress testing are baseline resolved from the parent analyte.

6. **Ruggedness:**

The ruggedness of an analytical method is the degree of reproducibility of test results Obtained by the analysis of the same samples under a variety of normal test conditions Such as different laboratories, different analysts, using operational and environmental Conditions that may differ but are still within the specified parameters of the assay. The Testing of the ruggedness is normally suggested when the method is to be used in more than One laboratory. Ruggedness normally expressed as the lack of the influence on the test results of operational and environmental variables of the analytical method.

For the determination or ruggedness, the degree of reproducibility of test result is determined as a function of the assay variable. This reproducibility may be compared to the precision of the assay under normal conditions to obtain a measure of the ruggedness of the analytical method.

7. Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as “a Measure of its capacity to remain unaffected by small, but deliberate variations in methodParameters”. A good practice is to vary important parameters in the method systematically and measure their effect on separation. The variable

method parameters in HPLC technique May involves flow rate, column temperature, sample temperature, pH and mobile phase composition.

8. Stability:

To generate reproducible and reliable results, the samples, standards, and Reagents used for the HPLC method must be stable for a reasonable time (e.g., one day, One week, one month, depending on need). Therefore, a few hours of standard and Sample solution suitability can required even for short (10 min) separation. When more than one sample is analyzed (multiple lots of one sample or samples from different Storage conditions from a single lot), automated, overnight runs often are performed for Better lab efficiency such practices add requirements for greater solution stability.

9. Limit of Detection:

Limit of detection (LOD) is the lowest concentration of analyte in a sample that can be Detected, but the necessarily quantitated, under the stated experimental conditions.

- Based on visual Evaluation
- Based on the standard Deviation of the Blank
- Based on the calibration curve
- Based on signal-to-noise: A signal-to-noise ratio of 3 or 2:1 is acceptable

LOD may be expressed as

$$LOD = 3.3\sigma/s$$

Where, σ = the standard deviation of the response

S= the slope of the calibration curve

The slope S may be estimated from the calibration of the analyte.

10. Limit of quantification:

Limit of quantification is the lowest concentration of analyte in a sample that can be Determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantification limit are possible.

- Based on visual Evaluation
- Based on standard Deviation of the blink
- Based on the calibration curve
- Based on the signal-to-Noise Approach: A typical signal-to-Noise is 10:1

LOQ may be expressed as

$$LOQ = 10\sigma/s$$

Where, σ = standard deviation of the response

S= the slope of the calibration curve[12]

2. DRUG PROFILE

PARACETMOL[19]:

Structure:

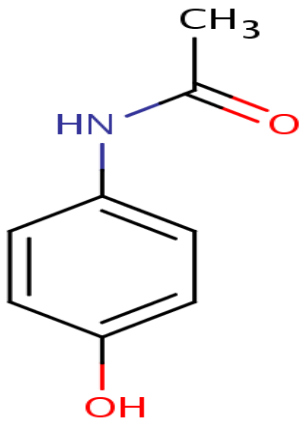


Fig No : 3 Structure of Paracetmol

Chemical name:

IUPAC Name **:** N-(4-hydroxyphenyl)acetamide

Molecular formula **:** $C_8H_9NO_2$

Molecular Weight **:** 151.1626gm/mol

Solubility **:** Soluble in water, methanol

CAS number **:** 103-90-2

Biological half life **:** 1 to 4 hours

Description:

Acetaminophen, also known as paracetamol, is commonly used for its analgesic and antipyretic effects. Its therapeutic effects are similar to salicylates, but it lacks anti-inflammatory, antiplatelet, and gastric ulcerative effects.

Mechanism of action:

Acetaminophen is thought to act primarily in the CNS, increasing the pain threshold by inhibiting both isoforms of cyclooxygenase, COX-1, COX-2, and COX-3 enzymes involved in prostaglandin (PG) synthesis. Unlike NSAIDs, acetaminophen does not inhibit cyclooxygenase in peripheral tissues and, thus, has no peripheral anti-inflammatory affects. While aspirin acts as an irreversible inhibitor of COX and directly blocks the enzyme's active site, studies have found that acetaminophen indirectly blocks COX, and that this blockade is ineffective in the presence of peroxides. This might explain why acetaminophen is effective in the central nervous system and in endothelial cells but not in platelets and immune cells which have high levels of peroxides. Studies also report data suggesting that acetaminophen selectively blocks a variant of the COX enzyme that is different from the known variants COX-1 and COX-2. This enzyme is now referred to as COX-3. Its exact mechanism of action is still poorly understood, but future research may provide further insight into how it works. The antipyretic properties of acetaminophen are likely due to direct effects on the heat-regulating centres of the hypothalamus resulting in peripheral vasodilation, sweating and hence heat dissipation.

Indications **:** For temporary relief of fever, minor aches, and pains.

Absorption **:** Rapid and almost complete

Protein binding : 25%

Metabolism : Acetaminophen primarily undergoes glucuronidation (45-55% of the dose) in which this process is facilitated by UGT1A1, UGT1A6, UGT1A9, UGT2B15 in the liver or UGT1A10 in the gut. 30-35% of the dose undergoes

sulfation. This biotransformation is facilitated by SULT1A1, SULT1A3, SULT1A4, SULT1E1 and SULT2A1. A small percentage of acetaminophen is oxidized by CYP2E1 to form N-acetyl-p-benzo-quinone imine (NAPQI), a toxic metabolite which is then conjugated to glutathione and excreted renally. Studies suggest that CYP3A4 and CYP2E1 are the primary cytochrome P450 isozymes responsible for the generation of toxic metabolites. Accumulation of NAPQI may occur if primary metabolic pathways are saturate.

Pharmacodynamics: Acetaminophen (USAN) or Paracetamol (INN) is a widely used analgesic and antipyretic drug that is used for the relief of fever, headaches, and other minor aches and pains. It is a major ingredient in numerous cold and flu medications and many prescription analgesics. It is extremely safe in standard doses, but because of its wide availability, deliberate or accidental overdoses are not uncommon. Acetaminophen, unlike other common analgesics such as aspirin and ibuprofen, has no anti-inflammatory properties or effects on platelet function, and it is not a member of the class of drugs known as non-steroidal anti-inflammatory drugs or NSAIDs. At therapeutic doses acetaminophen does not irritate the lining of the stomach nor affect blood coagulation, kidney function, or the fetal ductusarteriosus (as NSAIDs can). Like NSAIDs and unlike opioid analgesics, acetaminophen does not cause euphoria or alter mood in any way. Acetaminophen and NSAIDs have the benefit of being completely free of problems with addiction, dependence, tolerance and withdrawal. Acetaminophen is used on its own or in combination with pseudoephedrine, dextromethorphan, chlorpheniramine, diphenhydramine, doxylamine, codeine, hydrocodone, or oxycodone.

FLUPIRTINE MALEATE[20]:

Structure:

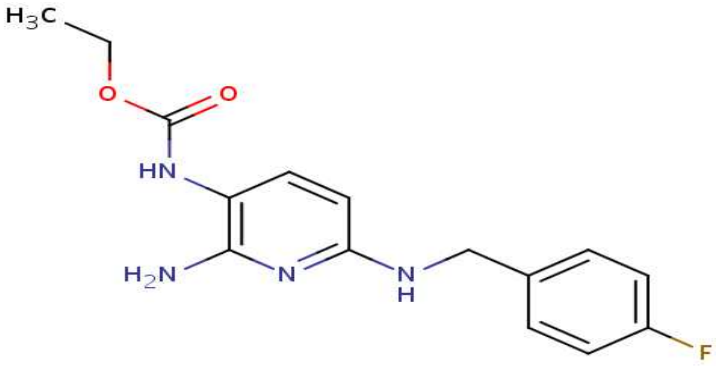


Fig No : 4 Stuctue of Flupirtine maleate

Chemical name:

IUPAC Name : Ethyl N-(2-amino-6-{[(4-fluorophenyl)methyl]amino}pyridin-3-yl) carbamate

Molecular formula : $C_{15}H_{17}FN_4O_2$

Molecular Weight : 304.3195 gm/mol

CAS number : 56995-20-1

Biological hal life : 6.5 hrs (average), 11.2-16.8 hrs (average 14 hrs) (elderly)

Solubility : Water soluble, organic solvents like ethanol. DMSO, Dimethylfomamide

Description :

Flupirtine is a pyridine derivative that is in clinical use as a nonopioid analgesic. It was approved for the treatment of pain in 1984 in Europe. It is not approved for use in the U.S. or Canada, but is currently in phase II trials for the treatment of fibromyalgia.

Mechanism of action:

Flupirtineupregulates Bcl-2, increases glutathione levels, activates an inwardly rectifying potassium channel, and delays loss of intermitochondrial membrane calcium retention capacity. Flupirtine acts like a NMDA receptor antagonists, but does not bind to the receptor. One study concluded that the discriminative effects of flupirtine are neither of opioid nor of alpha-1 adrenergic type, but are primarily mediated through alpha-2 adrenergic mechanisms [PMID: 2901483].

Indications : Investigated for use/treatment in fibromyalgia.

Absorption : Bioavailability: 90% (oral), 70% (rectal)

Metabolism: Hepatic to 2-amino-3-acetylamino-6-(para-fluorobenzylamino) pyridine (which has 20-30% the analgesic potential of its parent compound) and Para-fluorohippuric acid.

Route of elimination: 72% of flupirtine and its metabolites appear in urine and 18% appear in faeces.

3. LITERATURE REVIEW

1. K. V. Lalitha et al,[21] A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous estimation of Paracetamol (PARA) and Flupiritine Maleate (FLU) from pharmaceutical formulation. The method is carried out on Agilent C18 (25 cm x 4.6 mm i.d., 5 µ) column with a mobile phase consisting of Methanol : Water (0.2% TEA, adjusted to pH 3.0 using orthophosphoric acid) in the ratio of 90:10 v/v. The retention time of Paracetamol and Flupiritine Maleate is 3.2 min and 5.1 min respectively with the flow rate of 1mL/ min with VWD detection at 239 nm. The linear regression analysis data for the linearity plot showed good linear relationship with correlation coefficient value for Paracetamol and Flupiritine Maleate were R2=0.9995 and R2=0.9996 in the concentration range of 9-63 µg. mL-1 , 3-21 µg. mL-1 respectively. The relative standard deviation for intra-day precision has been found to be lower than 2.0 %. The method is validated according to the ICH guidelines. The developed method is validated in terms of specificity, selectivity, accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability. The proposed method can be used for simultaneous estimation of these drugs in marketed dosage forms.

2. **Umang Shah et al,[22]** Two methods for simultaneous estimation of Paracetamol and Flupirtine maleate in combineddosage form have been developed. The first method is the aplication of Q-analysis method (absorbance ratio),which involves the formation of Q-absorbance equation at 302 nm (isobestic point) and at 248 nm, themaximum absorbance of Paracetamol. The linearity ranges for PCM and FLU were 2-35 µg/ml and 0.5- 70µg/ml, respectively. The second method was based

on the use of first derivative spectroscopy, in whichderivative amplitudes were measured at selected wavelengths (234 nm ZCP of FLU for PCM and 34 nm ZCPof PCM for FLU), without mutual interference. The linearity ranges for PCM and FLU were 2-34 µg/ml and 2- 65 µg/ml, respectively. The acuracy of the methods were asesed by recovery studies and was found to be9.93% ±0.549 and 10.05% ±0.65 for Q absorbance ratio method and 9.54% ±0.591 and 9.41% ±0.792 forfirst derivative method, for PCM and FLU, respectively. These methods are simple, acurate and rapid; thoserequire no preliminary separation and thereforecan be used for routine analysis of both drugs in quality controllaboratories.

3. **Haritha et al,**[23]The present study describes a simple, accurate and precise RP-HPLC Technique for the simultaneous determination of Flupirtine maleate and Paracetamol in pharmaceutical dosage form. The method involves an isocratic elution of drug in a stationary phase of Phenomenex, C18 (150mm × 4.6mm, 5µm) column using a mobile phase composition of methanol and 0.1% (v/v) orthophosphoric acid in the composition ratio of 60:40 v/v with a flow rate of 0.8 mL/min at 270 nm of detection. The injection volume is 20 µL. the method has been validated for specificity, linearity, range, precision, accuracy, limit of detection, limit of quantification, ruggedness and robustness.The retention times for Flupirtine maleate and Paracetmol are about 3.07 and 4.63 minutes respectively. Quantitative linearity was observed over the concentration range of 10.08 to 302.51 µg/mL for Flupirtine maleate and 4.99 to 99.80 for Paracetamol respectively. The regression equations of concentration of Flupirtine maleate and Paracetamol are found to be y = 1774x+4755, y = 39182x + 64154 respectively where y is the peak area and x is the concentration of drug (µg/mL). The % recovery of Flupirtine maleate and Paracetamol are found to

be in the range of 97% to103 %. All the validation parameters are within the acceptance range

4. **GayatriGullipalli et al,**[24] A simple, specific, accurate, precise and economical reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous estimation of flupiritine maleate (FLU) and paracetamol (PARA) in bulk and pharmaceutical dosage form. The two components were separated using Inertsil ODS, C18 (250 mm × 4.6 mm id, 5 µm particle size) column by isocratic elution using mobile phase composition of potassium dihydrogen phosphate: methanol (70: 30) and pH 3.0 was adjusted with orthophosphoric acid. Flow rate used was 1ml/min and detection was carried out at 217nm. Injection volume is 5 µl. The retention time of flupiritine maleate and paracetamol is 2.553 min and 3.620 min respectively. As per ICH guidelines the method has been validated in terms of specificity, linearity, range, accuracy, precision, limit of detection, limit of quantitation, robustness. The method was found to be linear in the range of 50-150µg/ml(R2=0.999) for flupiritine maleate and 10-30 µg/ml (R2=0.999) for paracetamol. The limit of detection and limit of quantitation were found to be 2.14 and 7.13 for flupiritine maleate and 2.52 and 8.41 for paracetamol. Flupiritine maleate and paracetamol has the recoveries of 100.2% and 100.8% respectively and their relative standard deviations were less than 2%. All the validation parameters met the acceptance criteria. The proposed method can also be used for simultaneous estimation of these drugs in marketed dosage form.

5. **P. Giriraj et al,**[25]A new, simple, precise, accurate, reproducible, and efficient Vierordt's method or simultaneous equation method was developed and validated for simultaneous estimation of paracetamol and flupirtine maleate in pure and

pharmaceutical dosage form. The method was based on the measurement of absorbance at two wavelengths 245 nm and 344.5 nm, ofparacetamol and flupiritine maleate in 0.1 N HCl correspondingly. Calibration curves of paracetamol and flupiritine maleate were found to be linear in the concentration ranges of 5–15 µg/mL and 1.53–4.61 µg/mL, respectively, with their correlation coefficient values (R^2) 0.999. LOD and LOQ were 185.90 ng/mL and 563.38 ng/mL for paracetamol and 78.89 ng/mL and 239.06 ng/mL for flupiritine maleate. In the precision study, the % RSD value was found within limits (%). The percentage recovery at various concentration levels varied from 99.18 to 100.02% for paracetamol and 98.47 to 100.09% for flupiritine maleate confirming that the projected method is accurate. It could be concluded from the results obtained in the present investigation that this method for simultaneous estimation of paracetamol and flupirtine maleate in pure and tablet dosage form is simple, accurate, precise, and economical. The proposed method can be applied successfully for the simultaneous estimation of paracetamol and flupiritine maleate in pure and pharmaceutical dosage form.

6**. A.ALIEKYA et al[26],** This communication describe simple, sensitive, rapid, accurate, precise and cost effective First derivative spectrophotometric zero crossing method for the simultaneous determination of Paracetamol and Flupiritine Maleate in combined dosage form. The utility of first derivative data processing program is its ability to calculate unknown concentration of components of interest in a mixture containing an interfering component. The first order derivative absorption at 248 nm (zero cross point for Paracetamol) was used for estimation of Flupiritine Maleate and 254 nm (zero cross point for Flupiritine Maleate) was used for estimation of Paracetamol. Linear correlation was obtained between absorbance and concentrations of PARA and FLU in the concentration ranges of 2-14 µg/ml and 6.5-45.5 µg/ml, with

R2value 0.999 at both the wavelength respectively. The linearity of the calibrationcurve was validated by the high values of correlation coefficient of regression. The RSD values of PARA and FLU were found to be less than 2%. LOD and LOQ values for PARA were found to be 0.122 and 0.402µg/ml at 254 nm respectively. LOD and LOQ values for FLU were found to be 0.908 and 3.253 µg/ml at 248 nm, respectively.

7. **GollaMurali Mohan J et al,**[27]A simple, selective, rapid, precise and economical reverse phase high performance liquid chromatographic (RP-HPLC) method has been developed for the simultaneous estimation of Paracetamol (PARA) and Flupiritine Maleate (FLU) from pharmaceutical formulation. The method is carried out on Agilent C18 (25 cm x 4.6 mm i.d., 5 µ) column with a mobile phase consisting of Methanol : Water (0.2% TEA, adjusted to pH 3.0 using orthophosphoric acid) in the ratio of 90:10 v/v. The retention time of Paracetamol and Flupiritine Maleate is 3.2 min and 5.1 min respectively with the flow rate of 1mL/ min with VWD detection at 239 nm. The linear regression analysis data for the linearity plot showed good linear relationship with correlation coefficient value for Paracetamol and Flupiritine Maleate were R2=0.9995 and R2=0.9996 in the concentration range of 9-63 µg. mL-1 , 3-21 µg. mL-1 respectively. The relative standard deviation for intra-day precision has been found to be lower than 2.0 %. The method is validated according to the ICH guidelines. The developed method is validated in terms of specificity, selectivity, accuracy, precision, linearity, limit of detection, limit of quantitation and solution stability. The proposed method can be used for simultaneous estimation of these drugs in marketed dosage forms.

4. AIM & OBJECTIVE

Existing literature reveals that Paracetmol and Flupirtine maleate can be analyzed by HPLC using UV detection, TLC, HPTLC, HPLC in bulk material and pharmaceutical forms.

A comprehensive, validated and simple analytical method development and validation of Paracetmol and Flupirtine maleate tablets is, therefore, crucial. HPLC With PDA detector is a good selection as PDA detector is available in most laboratories, Therefore, in proposed project a successful attempt has been made to develop, simple, Accurate, and economic methods for analysis of Paracetmol and Flupirtine maleate tablets validated.

OBJECTIVE

The objective of the present work is to development and validate a HPLC method with PDA detector for the development and validation Paracetmol and Flupirtine maleate of tablets. To be employed in routine and stability tests. In the method development of Paracetmol and Flupirtine maleate we have decided to carry out our project work by incorporating the Reverse phase High performance Liquid chromatography (HPLC). Then the developed method will be validated according to ICH guidelines for its various Parameters.

5. PLAN OF WORK

In order to develop a simple, reliable and an accurate method development and validation of Paracetmol and Flupirtine maleatein pharmaceutical dosage form by Reverse phase HPLC and validate the method for its repeatability and reproducibility.

Plan of the proposed work includes the following steps:

- Selection of drug and literature survey.
- Solubility studies and optimization of conditions.
- Analytical method(s) development using HPLC etc.,
- Assay of the drugs(s) in marketed formulations using the proposed method(s).
- Procurement of raw materials.
- Establishment of system suitability parameters.
- Trails for the method development of Paracetmol and Flupirtine maleate.
- Setting of the optimized method.
- Validation of the optimized method for Paracetmol and Flupirtine maleate.

Validation parameters include:

- ❖ System suitability
- ❖ Specificity
- ❖ Method precision
- ❖ Linearity
- ❖ Accuracy
- ❖ Range
- ❖ Robustness

6. INSTRUMENTS/EQUIPMENT DETAILS

Instruments:

- WATERS HPLC, Model: Aliance 2695, Photo diode array detector (PDA), with anautomated sample injector. The output signal was monitored and integrated using Empower 2 software. AgelentC18 (4.6 x 250mm, 5 µm, Make: Waters) column was used for separations.

Table No.: 7 List of Equipments

S. No.	Equipment's	Software	Model	Company
1	Electronic Balance	NA	ER200A	ASCOSET
2	Ultra-Sonicator	NA	SE60US	ENERTECH
3	Heating Mantle	NA	BTI	BIO TECHNICS INDIA
4	Thermal oven	NA	---------	NARANG
5	pH Meter	NA	AD102U	ADWA
6	Filter Paper 0.45 microns	NA	---------	MILLI PORE

MATERIALS:

List of Chemicals and Reagents used

Table No. : 8 List of chemicals and reagents

S. No.	Chemicals/standards and reagents	Grade	Make
1	Ortho-Phosphoric Acid	AR	Finar
2	Methanol	HPLC	Merck
3	Water	HPLC	Loba Chemi
4	Paracetmol	NA	Dr. Reddy's
5	Flupirtine maleate	NA	Dr. Reddy's

7. METHOD DEVELOPMENT

TRAIL 1:

Mobile phase : Acetonitrile: methanol (50:50)

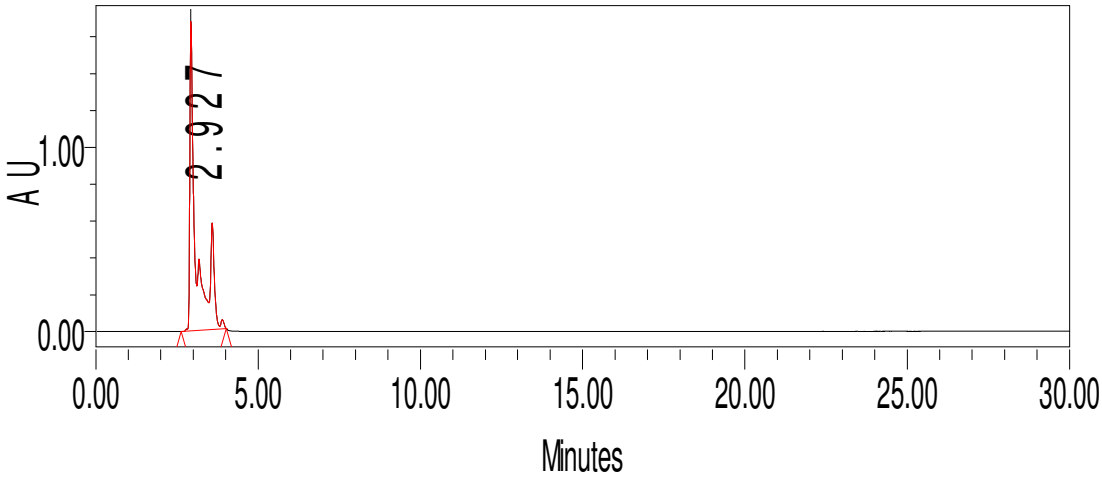
Auto sample temperature : $25^{o}c$

Injection volume : 10µL

Column : C18 Hypersil (250*4.6mm, 5µ)

Detector wavelength : 280nm

Flow rate : 1ml/min



	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1		2.927	22524731		6.76	3585

Figure No : 5 Typical chromatogram of trail 1

Observation : Peaks were not eluted

TRAIL 2:

Mobile phase : Acetonitrile: methanol (70:40)

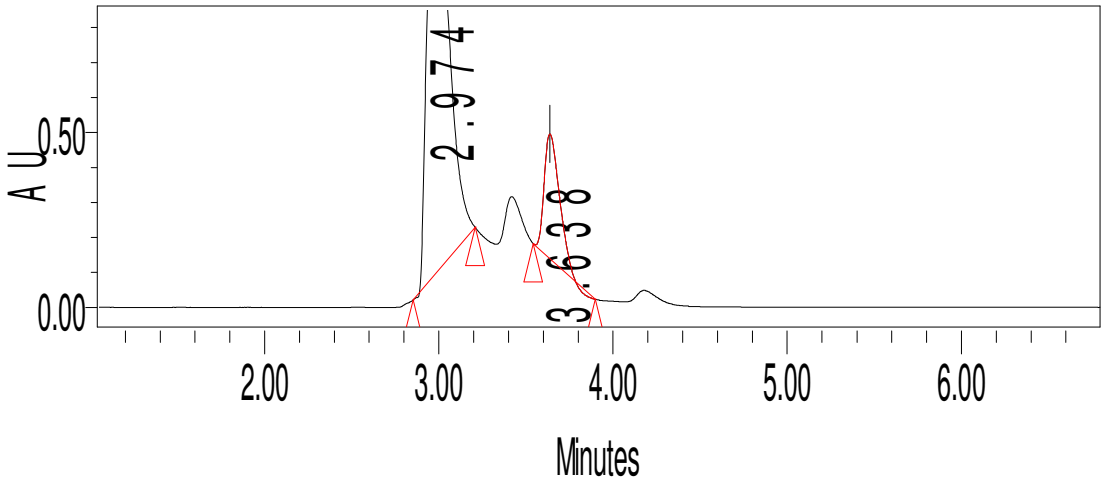
Auto sample temperature : 25°c

Injection volume : 10µL

Column : C18 Hypersil (250*4.6mm, 5µ)

Detector wavelength : 280nm

Flow rate : 1ml/min



	Name	Retention Time	Area	Height	USP Resolution	USP Tailing	USP Plate Count
2		3.638	2276685	356851	3.28	1.92	7325
1		2.974	9557650	1041912		1.69	2633

Figure no : 6 Typical chromatogram of trail 2

Observation: Peaks were not eluted

TRAIL 3:

Mobile phase : Acetonitrile: methanol (60:40)

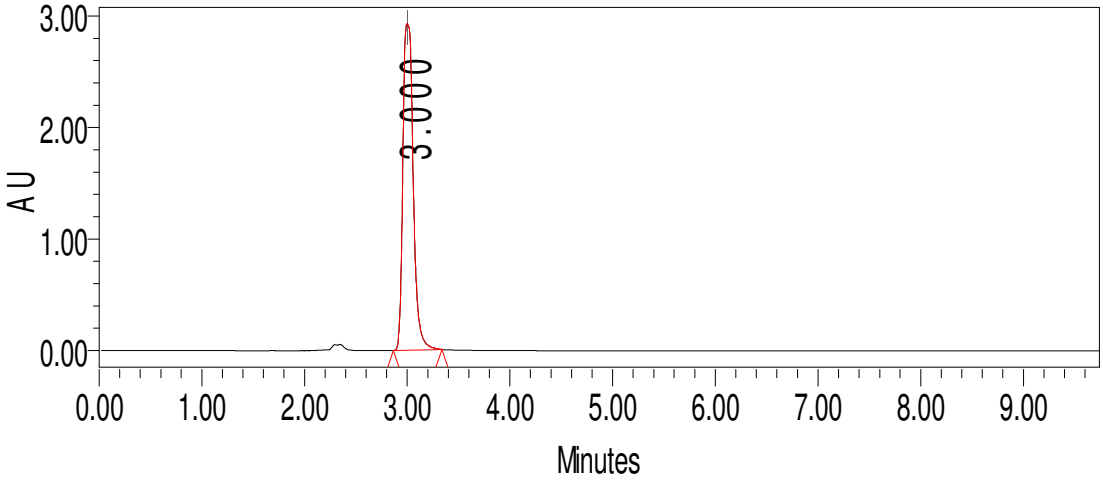
Auto sample temperature : $25^{o}c$

Injection volume : 10µL

Column : C18 Agilent (250*4.6mm, 5µ)

Detector wavelength : 280nm

Flow rate : 1ml/min



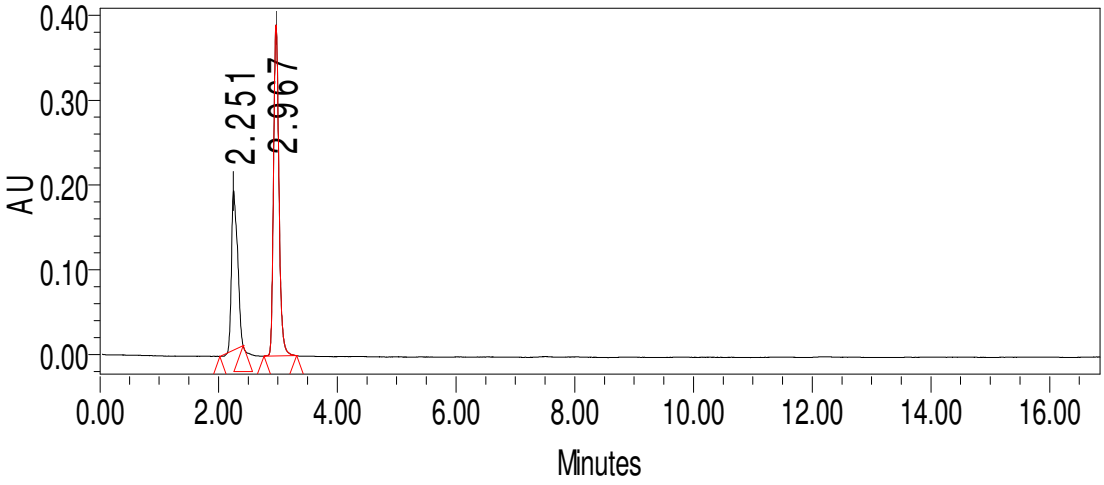
	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1		3.000	20493838		1.33	5781

Figure no: 7 Typical chromatogram of trail 3

Observation: Second peak was not eluted

TARIL: 4

Mobile phase	:	OPA buffer pH3.0: methanol (50:50)
Auto sample temperature	:	25°c
Injection volume	:	10µL
Column	:	C18 Agilent (250*4.6mm, 5µ)
Detector wavelength	:	280nm
Flow rate	:	1ml/min



	Name	Retention Time	Height	USP Resolution	USP Tailing	USP Plate Count
2		2.967	390401	4.03	1.23	5413
1		2.251	187896		1.31	2091

Figure no: 8 Typical chromatogram of trail 4

Observation: Two Peaks were eluted, but plate count fails.

TRAIL: 5

Mobile phase : OPA buffer pH3.0: methanol (80:20)

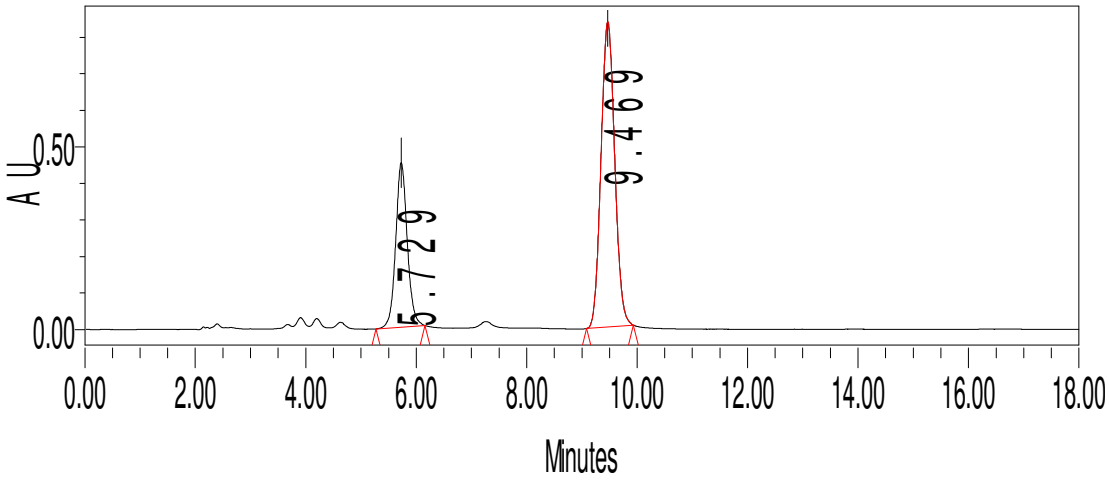
Auto sample temperature : 25°c

Injection volume : 10µL

Column : C18 Agilent (250*4.6mm,5µ)

Detector wavelength : 280nm

Flow rate : 0.8ml/min



	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
1		5.729	6539367		1.06	3723
2		9.469	14430602	8.81	1.07	6820

Figure No : 9 Typical chromatogram of trail 5

Observation : RT was found to be more

TRAIL: 6

Mobile phase : OPA buffer pH3.0: methanol (70:30)

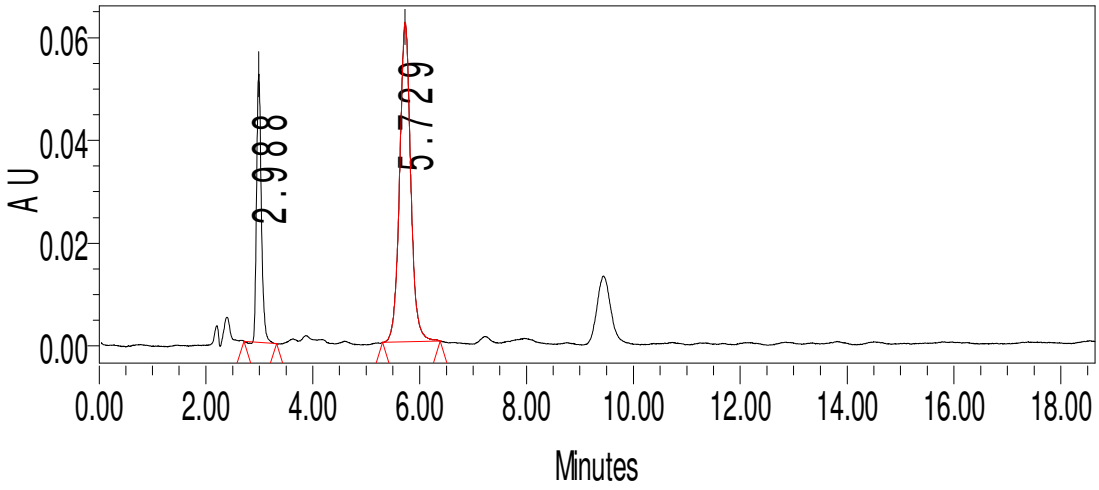
Auto sample temperature : 25°c

Injection volume : 10µL

Column : C18 Agilent (250*4.6mm,5µ)

Detector wavelength : 280nm

Flow rate : 0.8/min



	Name	Retention Time	Area	USP Resolution	USP Tailing	USP Plate Count
2		5.729	900158	10.06	1.04	3836
1		2.988	327285		1.28	5532

Figure no: 10 Typical chromatogram of trail 6

Observation: RT was found to be more

TRAIL 7:

Mobile phase : OPA buffer pH3.0: methanol (65:35)

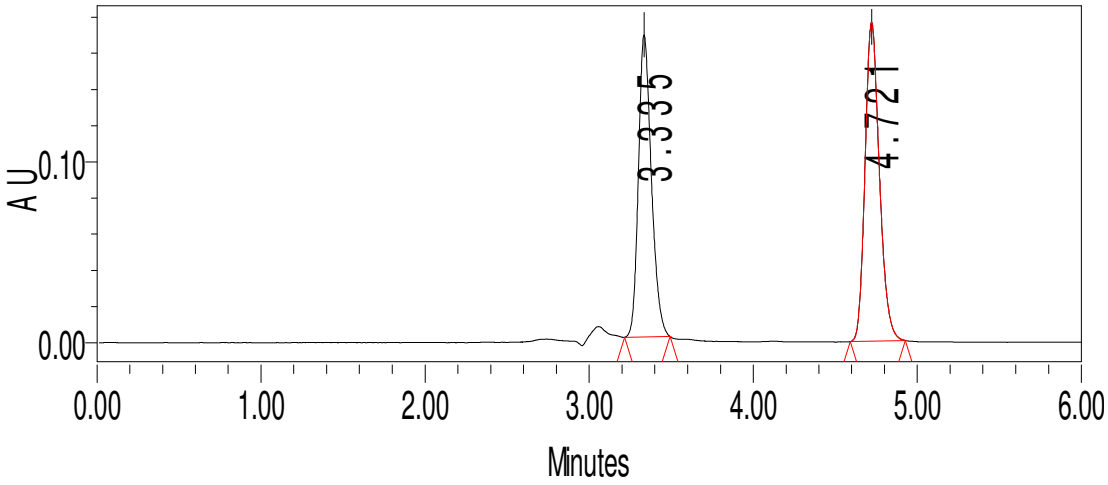
Auto sample temperature : 25°c

Injection volume : 10µL

Column : C18 Agilent (250*4.6mm,5µ)

Detector wavelength : 280nm

Flow rate : 0.8/min



	Name	Retention Time	Area	End Time	USP Resolution	USP Tailing	USP Plate Count
2		4.721	1078997	4.941	8.864	1.21	13930
1		3.335	920750	3.503		1.25	8503

Figure no: 11 Typical chromatogram of trail 7

Observation: RT was found to be more

8. OPTIMIZED METHOD

Chomatographic parameters:

Mobile phase : OPA buffer pH3.0: methanol(65:35)

Auto sample temperature : 25°c

Injection volume : 10µL

Column : C18AgIlent(250*4.6mm,5µ)

Detector wavelength : 280nm

Flow rate : 0.8ml/min

Procedure:

Inject 10µL of standard, sample into chromatographic system and measure the areas for the Paracetmol and Flupirtine maleate peeks and calculate the % assay by using the formula

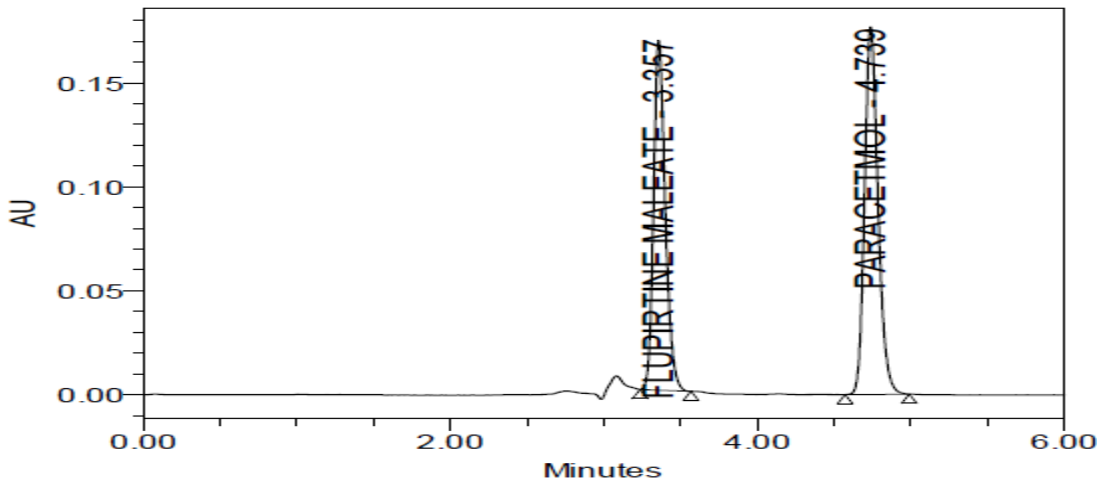


Fig No:12 Chromatogram for optimized method

Observation: Peeks are well separated all the parameters are within the limits.

PREPARATION OF MOBILE PHASE:

Transfer 1000ml of HPLC water into 1000ml of beaker and adjust pH 3.0 with orthophosphoricacid(OPA).

Transfer the above solution 650ml and 350ml of buffer and methanol is used as mobile phase. They are mixed and sonicated for 20min.

PREPARATION OF THE PARACETMOL AND FLUPIRTINE MALEATESTANDARD AND SAMPLE SOLUTION:

PREPARATION OF STANDARD SOLUTION:

Accurately weigh and transfer 325mg of Paracetmol and 100mg of Flupirtine maleate into 100ml of volumetric flask and add 10ml of Methanol and sonicate 10min (or) shake 5min and make with water. Transfers the above solution into 5ml into 25ml volumetric flask dilute to volume with water.

PREPARATION OF SAMPLE STOCK SOLUTION:

Commercially available 20 tablets ware weighed and powdered the powdered equivalent to the 802.04mg of Paracetmol and Flupirtine maleate of active ingredients were transfer into a 100ml of volumetric flask and add 10ml of Methanol and sonicate 20min (or) shake 10min and makeup with water.

Transfers above solution 5ml into 25ml of the volumetric flask dilute the volume with Methanol. And the solution was filtered through 0.45µm filter before injecting into HPLC system.

9. METHOD VALIDATION

1. SYSTEM SUITABILITY:

Tailing factor for the peaks due to Paracetmol and Flupirtine maleatein standard solution should not be more than 2.0.Theoretical plates for the Paracetmol and Flupirtine maleate peaks in standard solution should not be less than 2500.

2. SPECIFICITY:

Solution of standard sample and placebo were prepared as per test procedure and injected into the HPLC system.

Acceptance criteria:

Chromatogram of standard and sample should be identical with near Retention time.

Blank interference:

A study to establish the interference of blank was conducted. Diluent was injected into HPLC system as per the test procedure.

Acceptance criteria:

Chromatogram of blank should not show any peak at the retention time of analytepeak. There is no interference due to blank at the retention time of analyte. Hence the method is specific.

3. LINEARITY

Prepare a series of standard solutions and inject into HPLC system. Plot the graph of standard versus the actual concentration in µg/ml and determine the coefficient of correlation and basis for 100% response.

Acceptance criteria:

Linearity regression coefficient of average peak area response of replicate injections plotted against respective concentration should not be less than 0.999. The % y-intercept as obtained from the linearity data (without extrapolation through origin 0, 0) should be within ±2.0.

Statistical Evaluation:

A graph between the concentration and the average area was plotted. Points for linearity were observed. Using the method of least squares, a line of best fit was taken and the correlation Coefficient, slope and, y-intercept were calculated.

4. PRECISION:

Preparation of sample:

- Transfer the 802.04mg of sample into a 100ml of volume at flask and add 10ml of water and 10ml of Methanol andsonicate 20min and makeup with water. Transfer the above solution into 5ml into 25ml volume metric flask dilute to the volume with water.
- The method precision parameters were evaluated from sample chromatograms obtained, by calculating the % RSD of peek areas from 6 replicate injection.

Acceptance criteria: The injection reproducibility requirements are met if the %RSD for peak areas is not more than 2.0 and for retention times is not more than 2.0.

5. RECOVERY/ACCURACY

Recovery study can be performed in the concentration range of 80%, 100% to 120% of the target concentration of the test. Minimum 3 concentrations are recommended.

Acceptance criteria:

The average percentage recovery was between 97-103% and Relative standard deviation of these recovery concentrations was less than 2%.

6. LIMIT OF DETECTION

The sensitivity of measurement of Paracetmol and Flupirtine maleateby use of proposed method was estimated in terms of the limit of detection (LOD). The LOD was calculated calculated by the use of signal to noise ratio. In order to estimate the LOD value, the blank sample was injected six times and peak area of this blank was calculated as noice level. The LOD was calculated as three times the noise level.

$$LOD= 3.3 \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

7. LIMIT OF QUANTIFICATION:

The sensitivity of measurement of Paracetmol and Flupirtine maleateby the use of proposed method was estimated in terms of limit of quantification (LOQ). The LOQ was calculated by the use of signal to noise ratio. In order to estimate the LOQ value, the blank sample was injected six times and thepeak area of this blank was calculated at noise level. TheLOQ was calculated as ten times the noise value gave the LOQ.

$$LOQ = 10 \sigma / S$$

Where,

σ = standard deviation of intercepts of calibration curves

S = mean of slopes of the calibration curves

The slope S may be estimated from the calibration curve of the analyte.

8.ROBUSTNESS:

Effect of variation in flow rate:

Prepare the system suitability solution as per the test method and inject into the HPLC system with ±0.2 ml of the method flow. Evaluate the system suitability values as required by the test method for both flow rates. Actual flow rate was 1.0 ml/min and it was changed to 0.8ml/min and 1.2ml/min and inject into HPLC and system suitability was checked.

Effect of variation in Temperature:

Prepare the system suitability solution as per the test method and injected into the HPLC with ±5^{o}c of the method temperature. Evaluate the system suitability values as required by the test method for both temperatures.

10. SYSTEM SUITABILITY

Table No: 9 System suitability data of Paracetmol and Flupirtine maleate

Parameter	Paracetmol	Flupirtine maleate	Acceptance criteria
Retention time	4.731	3.353	
Theoretical plates	13958	9156	>2500
Tailing factor	1.24	1.29	<2.00
% RSD	0.1	0.1	<2.00

Table No : 10 Standard Results of Paracetmol

S. No.	Sampl name	RT	Area	USP plate count	USP tailing
	Injection1	4.720	1081754	13904	1.22
	Injection 2	4.739	1089163	13901	1.23
	Injection 3	4.716	1097378	13879	1.23
	Injection 4	4.736	1084949	14257	1.23
	Injection 5	4.738	1089191	14143	1.22

Table No : 11 Standard Results of Flupirtine maleate

S.No	Sample name	RT	Area	USP plate count	USP tailing
1.	Injection 1	3.333	928391	8453	1.27
2.	Injection 2	3.360	904815	8979	1.26
3.	Injection 3	3.342	906819	9188	1.26
4.	Injection 4	3.358	909101	9228	1.27
5.	Injection 5	3.354	907622	9298	1.27

Fig No: 13 typical Chromatogram of Standard 1

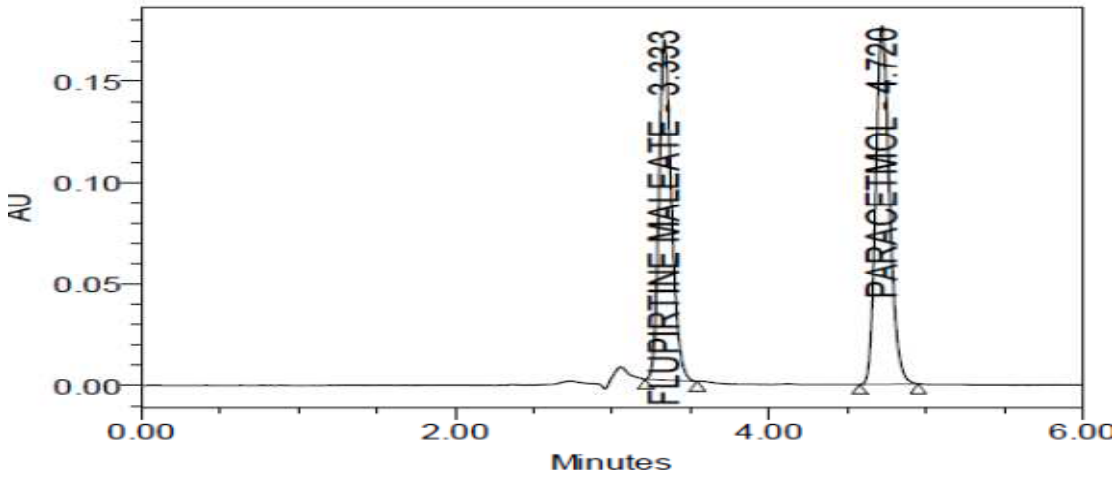


Fig No: 14 typical Chromatogram of Standard-2; Injection-1

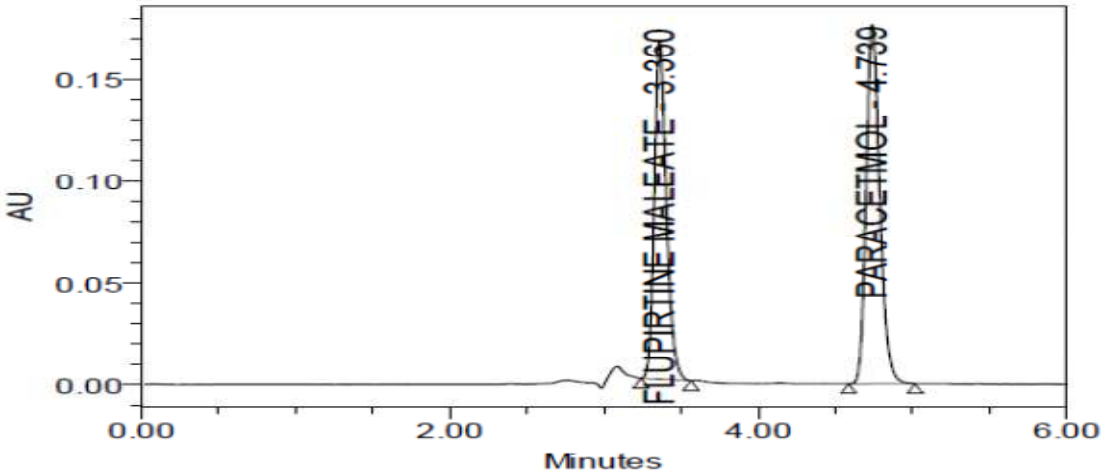


Fig No: 15 typical Chromatogram of Standard-2; Injection-2

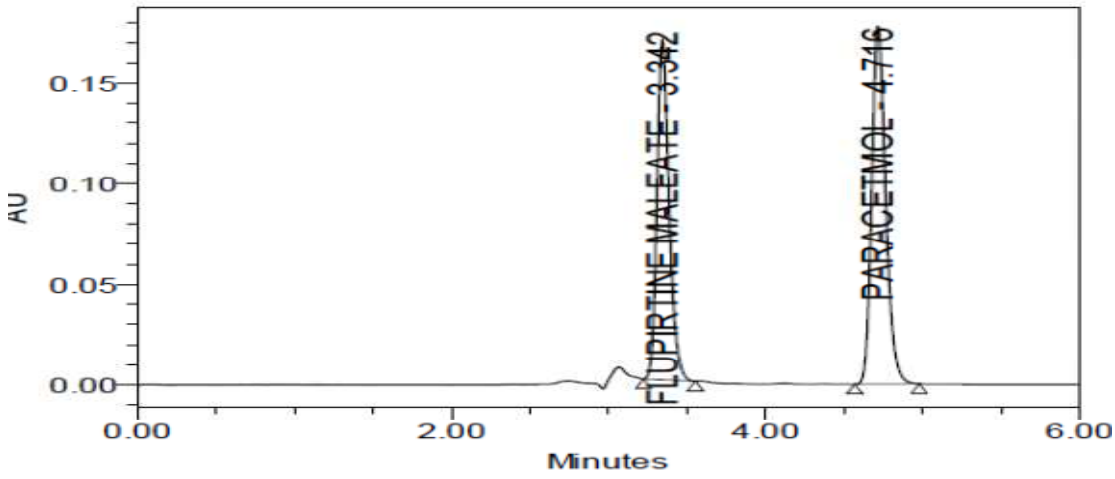


Fig No: 16 typical Chromatogram of Standard-2; Injection-3

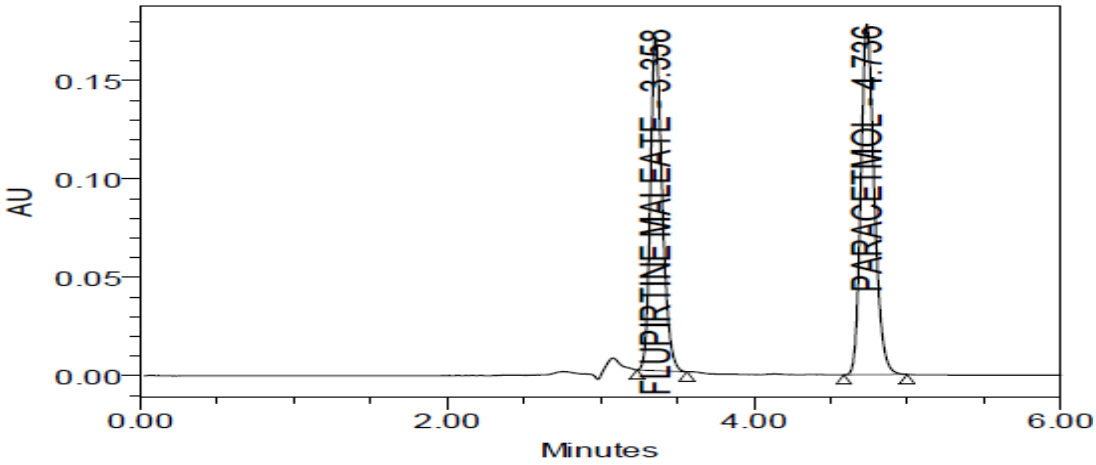


Fig No: 17 typical Chromatogram of Standard-2; Injection-4

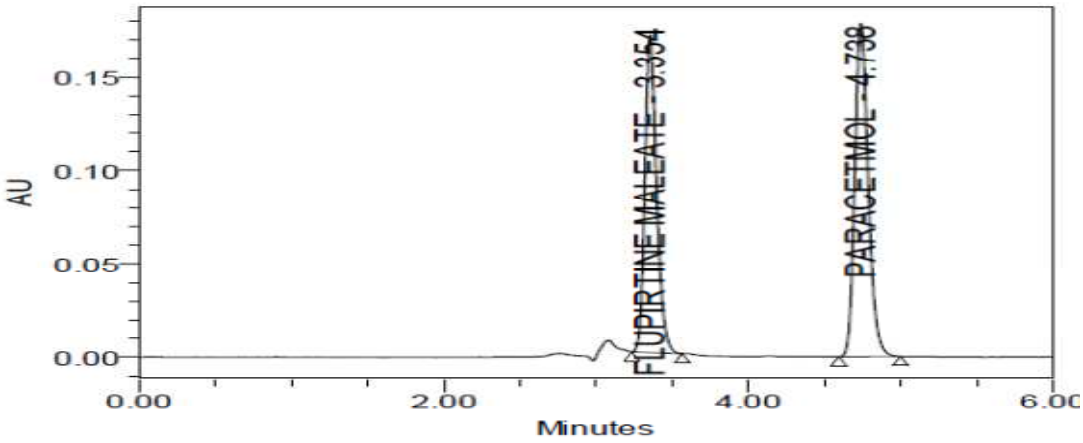


Fig No: 18 typical Chromatogram of Standard-2; Injection-5

RESULT

Results of system suitability study are summarized in the above table. Six consecutive injections of the standard solution showed uniform retention time, theoretical plate count, tailing factor and resolution for both the drugs which indicate a good system for analysis.

2. SPECIFICITY:

Table no: 12 Specificity data for Paracetmol and Flupirtine maleate

S no	Sample name	Paracetmol area	Rt	Flupirtine maleate Area	Rt
1	Standard	2251240	1.950	21020626	3.858
2	Sample	2252365	1.944	21107354	3.838
3	Blank	-	-	-	-
4	Placebo	-	-	-	-

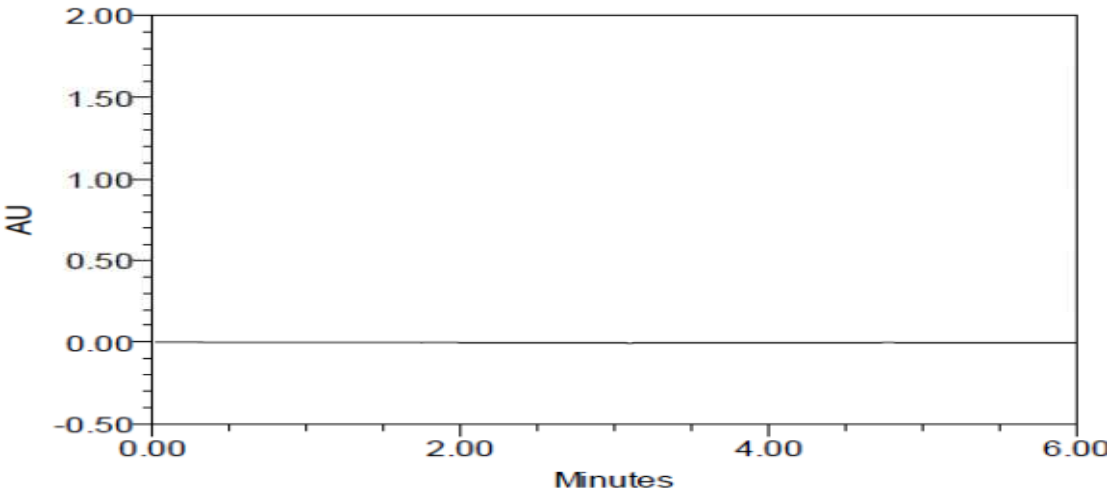


Fig no: 19 Typical chromatogram of the blank

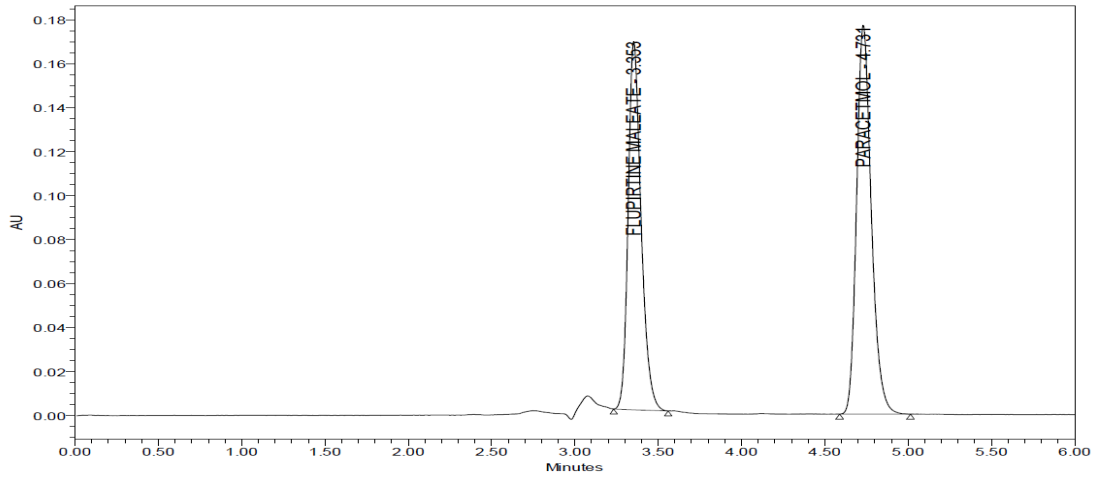


Fig no: 20 chromatogram representing specificity of standard

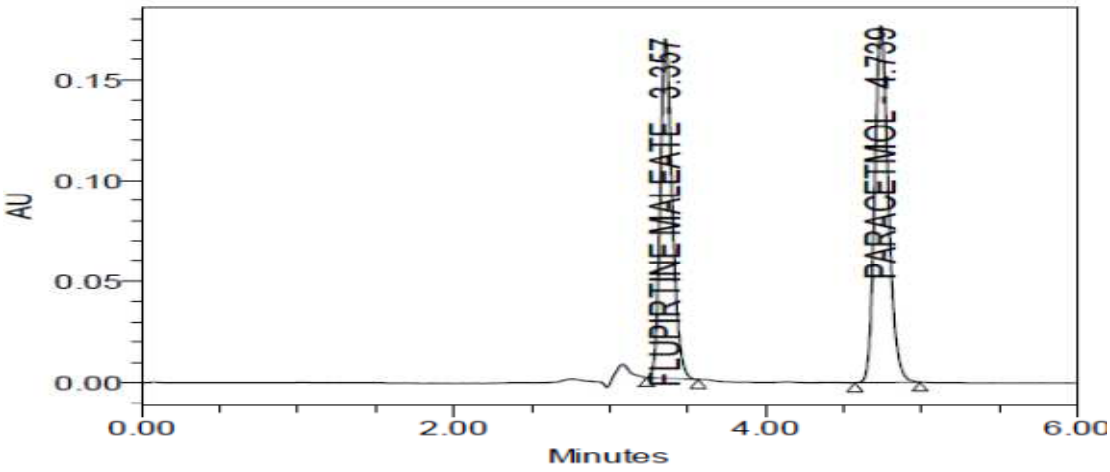


Fig no: 21 chromatogram representing specificity of sample

RESULT

Chromatograms explain that retention time for standard, sample and commercial product of Paracetmol and Flupirtine maleate are same. This proves that, excipients have no effect on the analytical method. On the other hand, blank peak did not overlap drug peak. So the method is highly selective.

3. ACCURACY:

Table No: 13 Accuracy data for Paracetmol

S.NO	Accuracy level	injecton	Sample area	RT
1	80%	1	541598	4.733
		2	546689	4.726
		3	541728	4.709
2	100%	1	1089965	4.728
		2	1080210	4.732
		3	1085854	4.715
3	120%	1	1667553	4.755
		2	1666002	4.743
		3	1666117	4.732

Table no: 14 Accuracy (%recovery) results of Paracetmol

S.NO	Accuracy Level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	80%	1	401.02	321.750	322.45	100	101
		2	401.02	321.750	325.48	101	
		3	401.02	321.750	322.53	100	
2	100%	1	802.04	643.500	648.93	101	100
		2	802.04	643.500	643.12	100	
		3	802.04	643.500	646.48	100	
3	120%	1	1203.06	965.250	992.81	103	103
		2	1203.06	965.250	991.88	103	
		3	1203.06	965.250	991.95	103	

Table no: 15 Accuracy data for Flupirtine maleate

S.NO	Accuracy level	Sample name	Sample area	RT
1	80%	1	459327	3.348
		2	456469	3.358
		3	450190	3.335
2	100%	1	910952	3.345
		2	916393	3.366
		3	916445	3.337
3	120%	1	1367985	3.370
		2	1364944	3.367
		3	459327	3.348

Table no: 16 Accuracy (%recovery) results of Flupirtine maleate

S.NO	Accuracy level	Sample name	Sample weight	µg/ml added	µg/ml found	% Recovery	% Mean
1	80%	1	401.02	99.000	99.89	101	100
		2	401.02	99.000	99.27	100	
		3	401.02	99.000	97.91	99	
2	100%	1	802.04	198.000	198.11	100	100
		2	802.04	198.000	199.30	101	
		3	802.04	198.000	199.31	101	
3	120%	1	1203.06	297.000	297.51	100	100
		2	1203.06	297.000	296.85	100	
		3	1203.06	297.000	296.15	100	

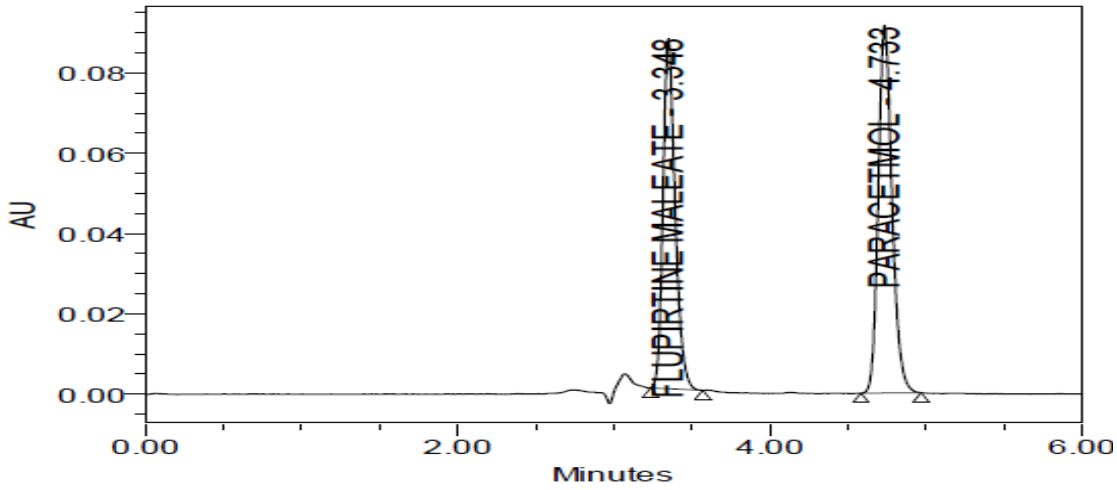


Fig no: 23 Typical chromatogram for Accuracy 80 %

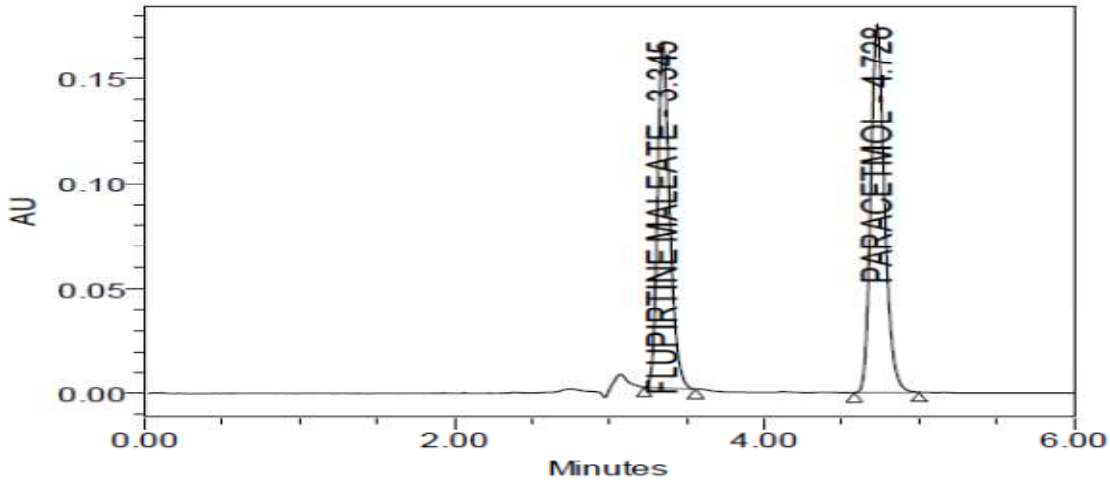


Fig no: 24 Typical chromatogram for Accuracy 100 %

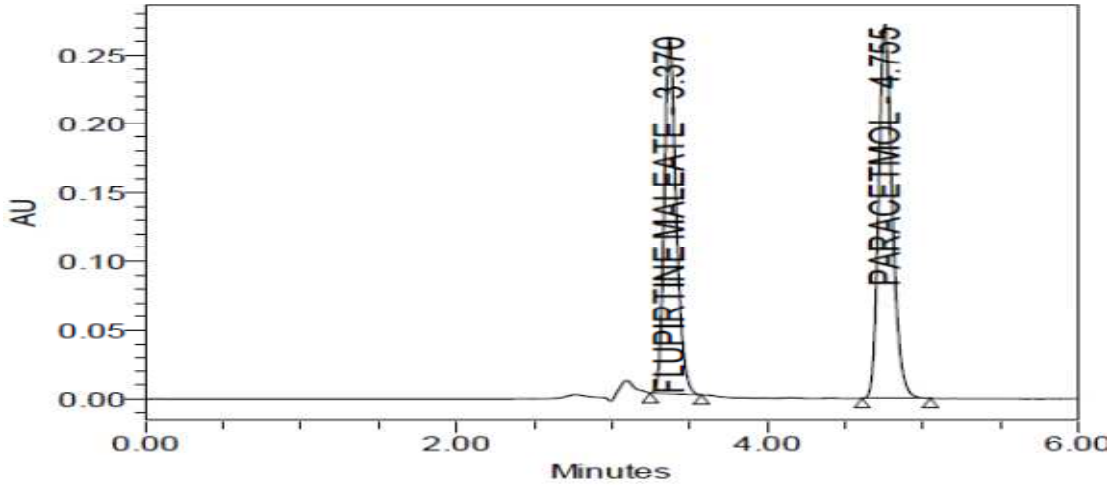


Fig no: 25 Typical chromatogram for Accuracy 120 %

RESULT

Results of accuracy study are presented in the above table. The measured value was obtained by recovery test. Spiked amount of both the drug were compared against the recovery amount.

% Recovery was 101.00% for Paracetmol and 100.00% for Flupirtine maleate. All the results indicate that the method is highly accurate**.**

PRECISION:

Table no: 17 Precision data for Paracetmol

S.no	RT	Area	%Assay
injection1	4.739	1078871	99
injection2	4.734	1064983	98
injection3	4.746	1083323	99
injection4	4.727	1080612	99
injection5	4.725	1078487	99
injection6	4.740	1072778	98
Mean			99
Std. Dev.			0.61
% RSD			0.62

Table no: 18 Precision data for Flupirtine maleate

S.no	RT	Area	%Assay
injection1	3.357	911468	99
injection 2	3.356	910692	99
injection 3	3.365	913771	99
injection 4	3.354	914557	99
injection 5	3.342	910623	99
injection 6	3.359	910986	99
Mean			99
Std. Dev.			0.19
%RSD			0.19

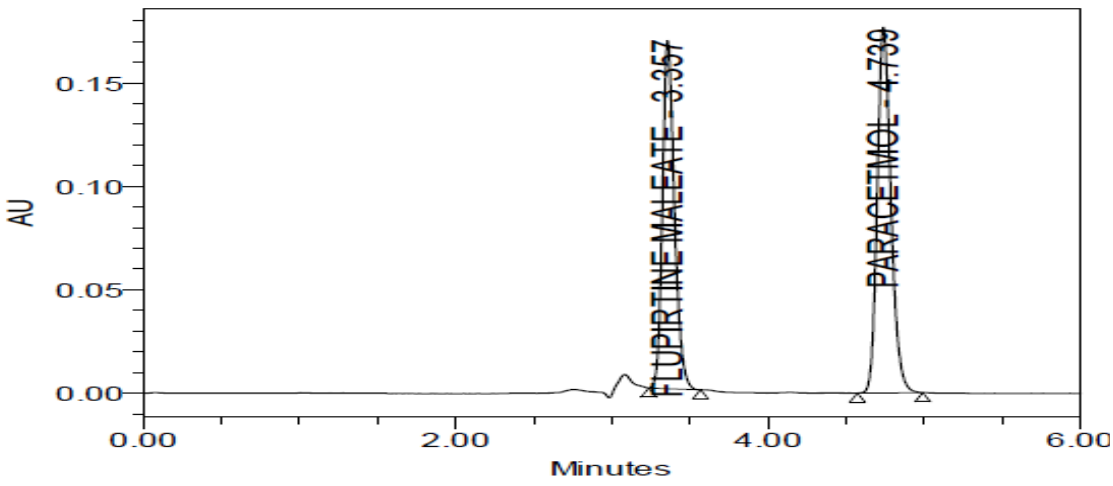


Fig no: 26 Chromatogram for precision injection 1

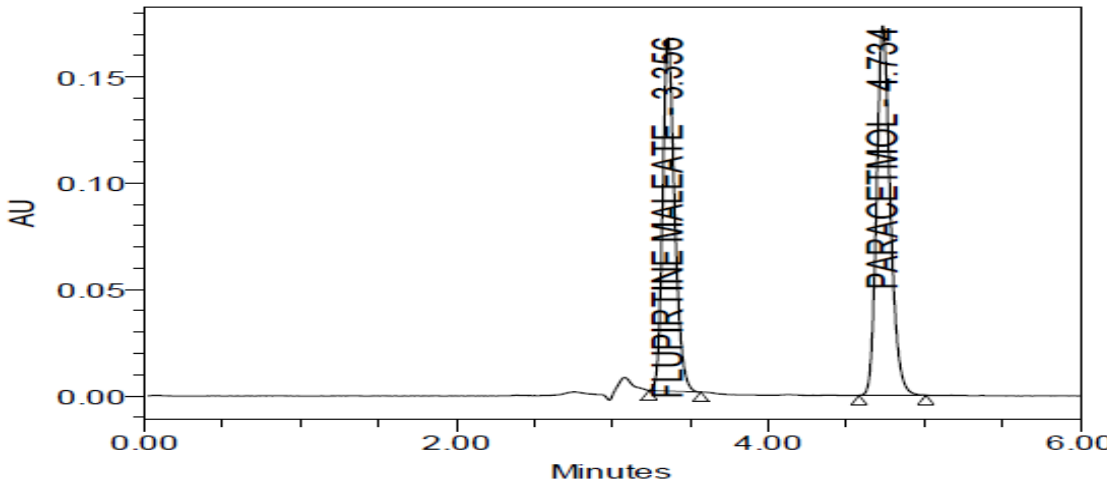


Fig no: 27 Chromatogram for precision injection

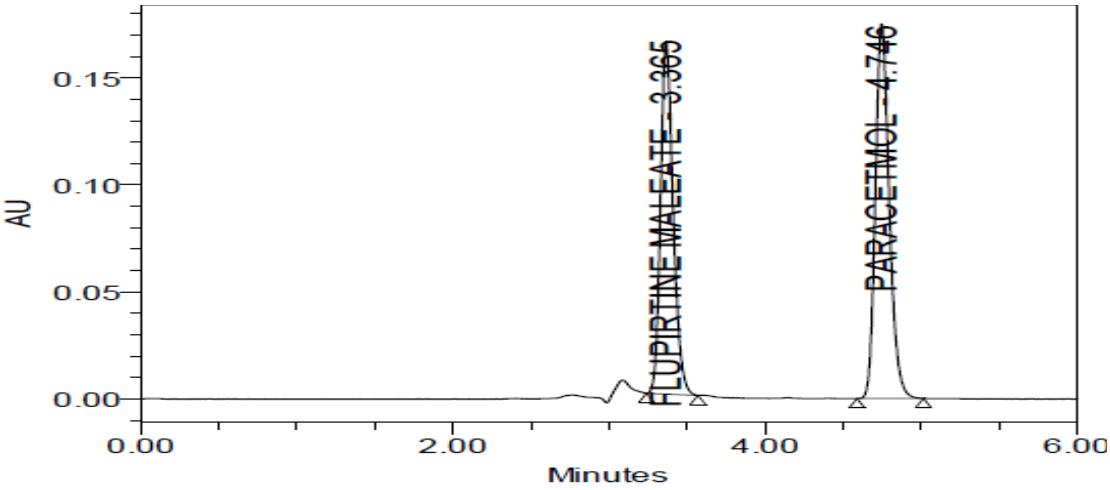


Fig no: 28 Chromatogram **for precision injection 3**

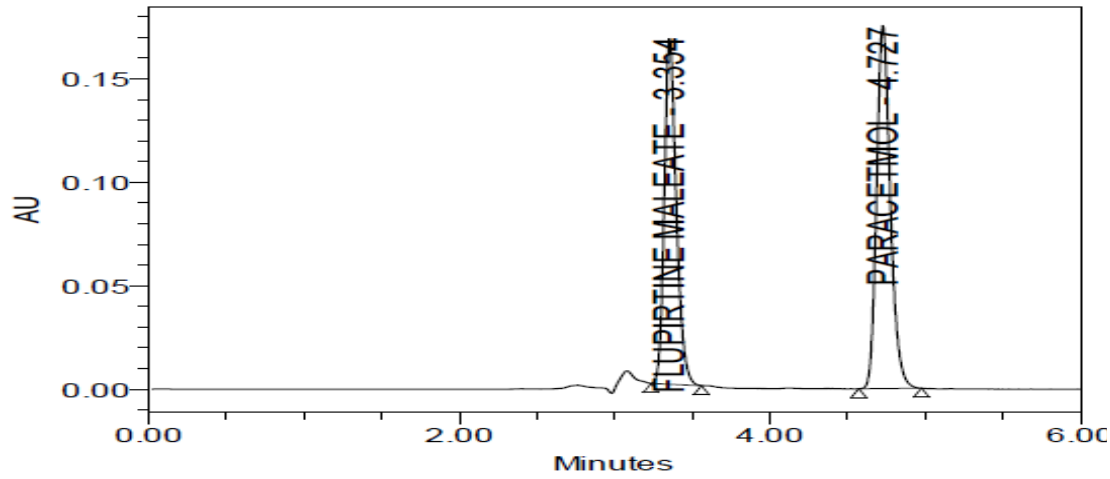


Fig no: 29 Chromatogram for precision injection 4

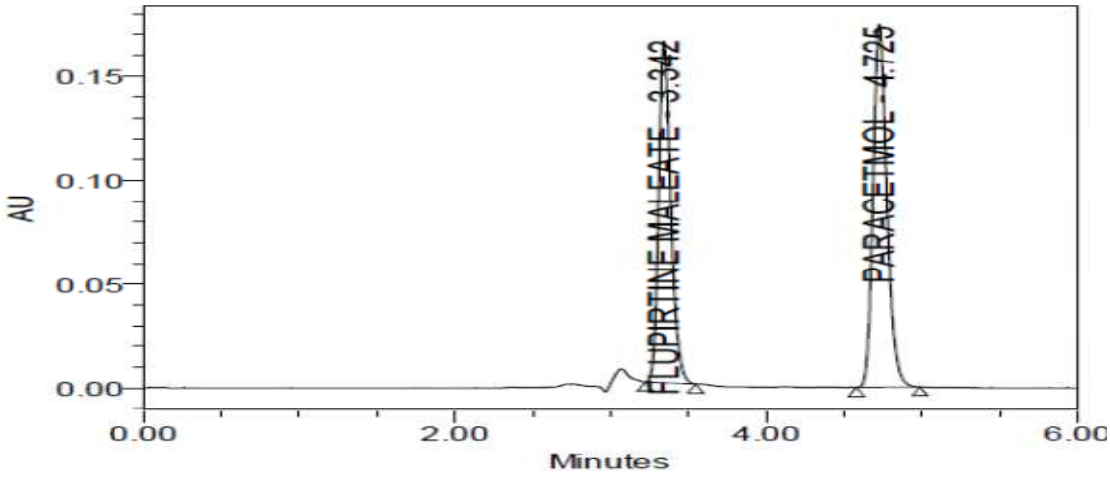


Fig no: 30 Chromatogram for precision injection 5

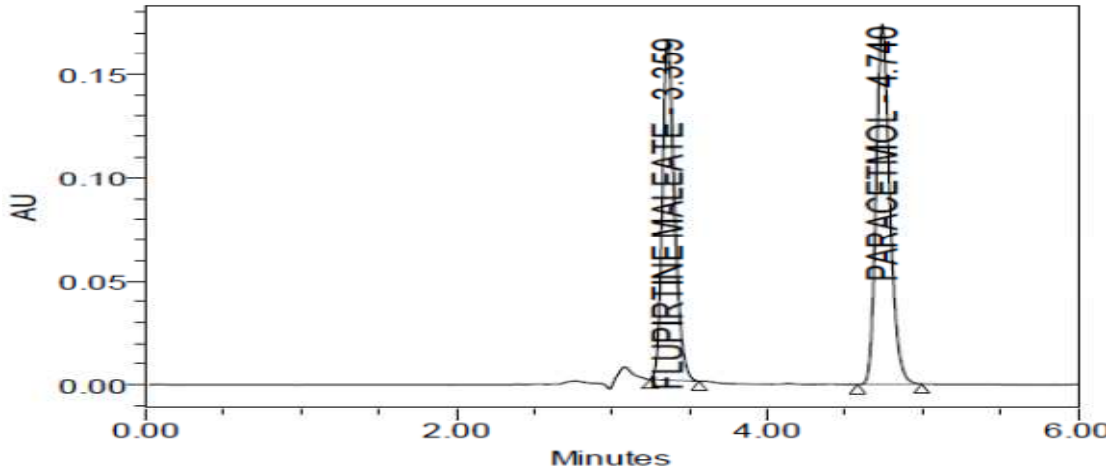


Fig no: 31 Chromatogram for precision injection 6

RESULT

Results of variability were summarized in the above table. % RSD of peak areas was calculated for various run. Percentage relative standard deviation (%RSD) was found to be less than 2% which proves that method is precise**.**

5. LINEARITY:

Table no: 19 Linearity data for Paracetmol

S.no	Conc(µg/ml)	RT	Area
1.	50	4.741	545934
2.	75	4.745	545471
3.	100	4.738	1089953
4.	125	4.733	1363163
5.	150	4.729	1646171

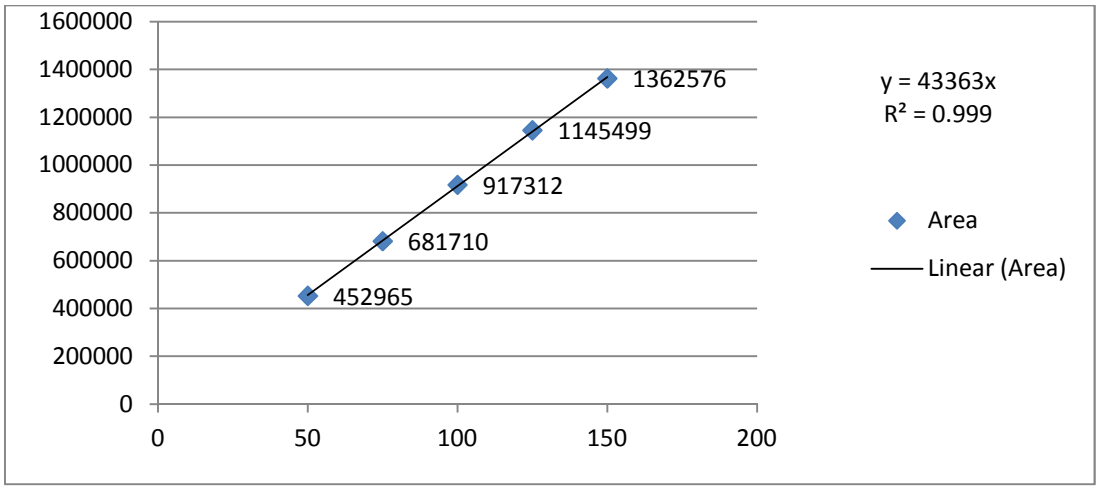


Fig no: 32 Linearity plot of Paracetmol

Table no: 20 Linearity data for Flupirtine maleate

s.no	Conc(µg/ml)	RT	Area
1.	50	3.362	452965
2.	75	3.359	681710
3.	100	3.359	917312
4.	125	3.357	1145499
5.	150	3.356	1362576

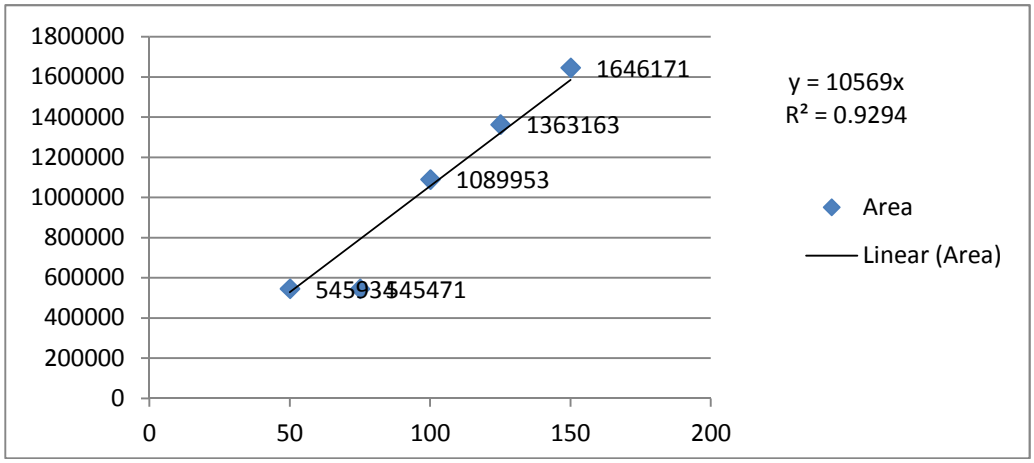


Fig no: 33 Linearity plot of Flupirtine maleate

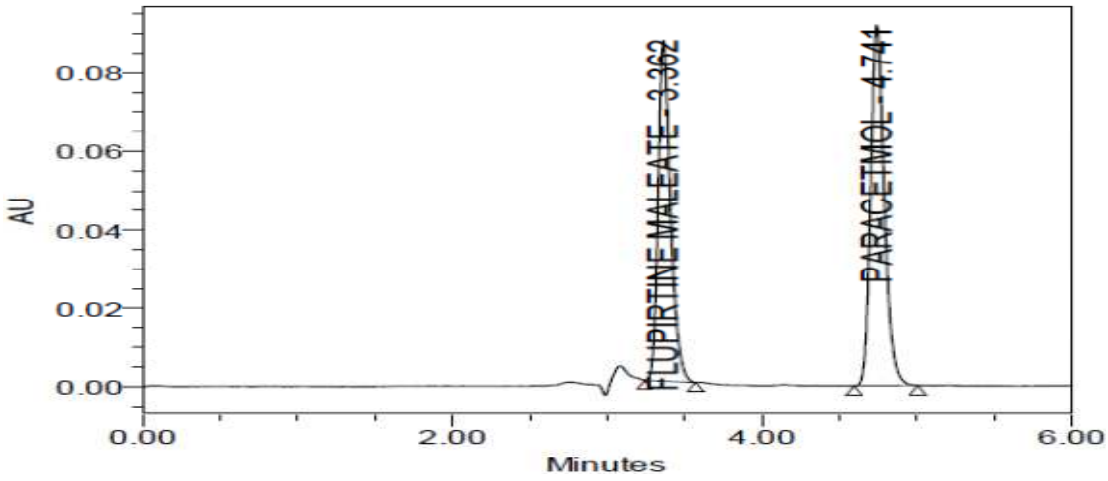


Fig no: 34 Chromatogram representing lineartity 1

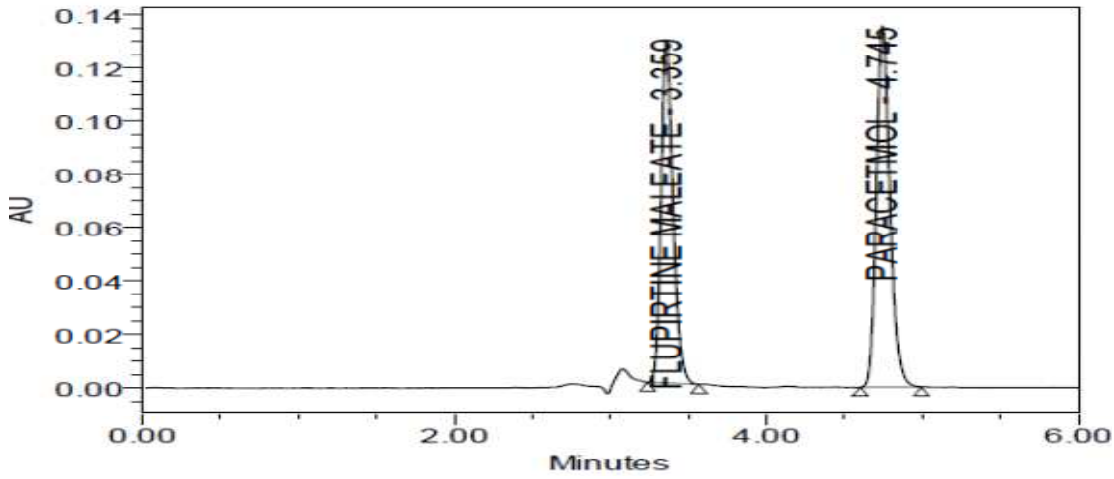


Fig no: 35 Chromatogram representing linearity 2

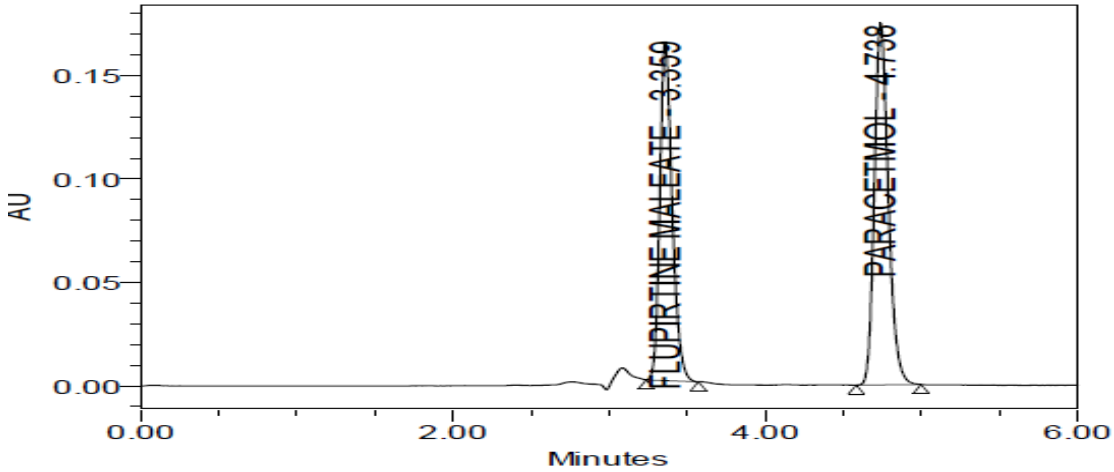


Fig no: 36 Chromatogram representing linearity 3

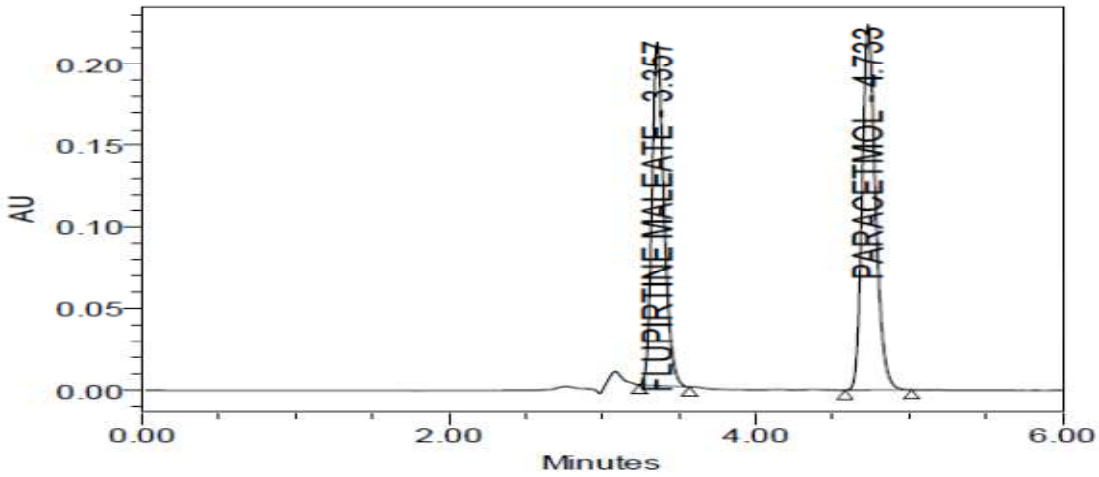


Fig no: 37 Chromatogram representing linearity 4

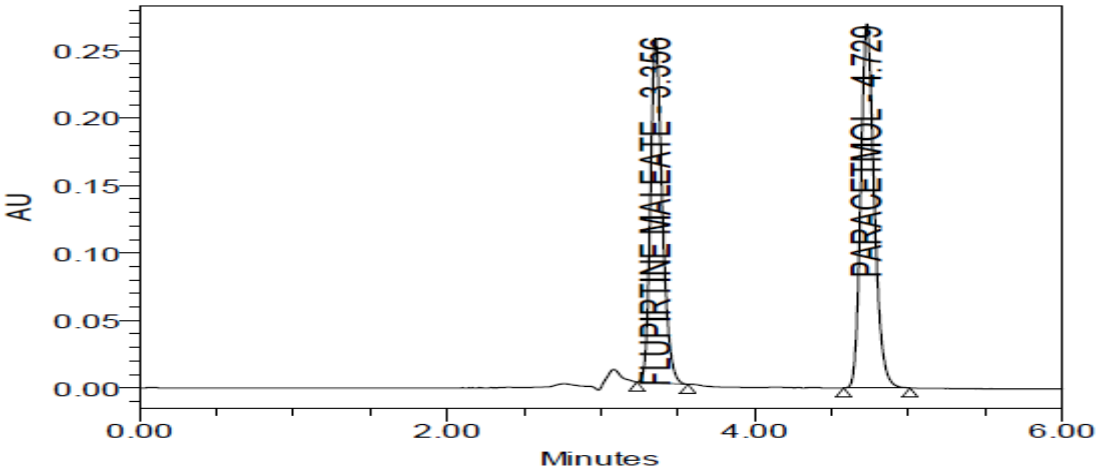


Fig no: 38 Chromatogram representing linearity 5

RESULT

A linear relationship between peak areas versus concentrations was observed for Paracetmol and Flupirtine maleate in the range of 50% to 150% of nominal concentration. Correlation coefficient was 0.999 for both Paracetmol and Flupirtine maleate which prove that the method is linear in the range of 50% to 150%.

6. ROBUSTNESS:

Table no: 21 Robustness data for Paracetmol

parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.6ml/min)	5.270	14731	1.27
Increased flow rate(1.0ml/min)	4.321	13704	1.21
Decreased temperature(20^0c)	5.237	13997	1.25
Increased temperature(30^0c)	4.310	13146	1.22

Table no: 22 Robustness data for Flupirtine maleate

parameter	RT	Theoretical plates	Asymmetry
Decreased flow rate(0.6ml/min)	3.726	9384	1.31
Increased flow rate(1.0ml/min)	3.050	8891	1.26
Decreased temperature(20^0c)	3.710	9499	1.31
Increased temperature(30^0c)	3.049	9005	1.26

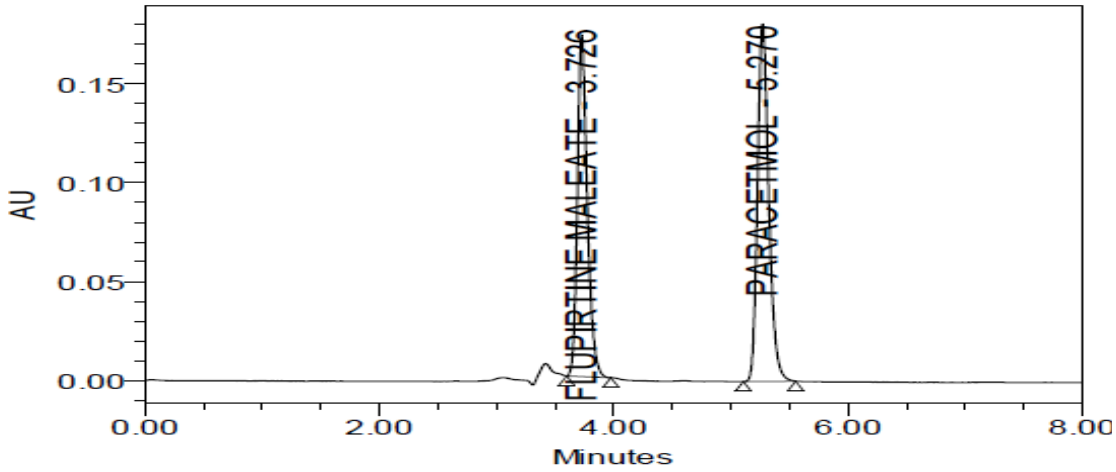


Fig no: 39 Chromatogram for decreased flow rate

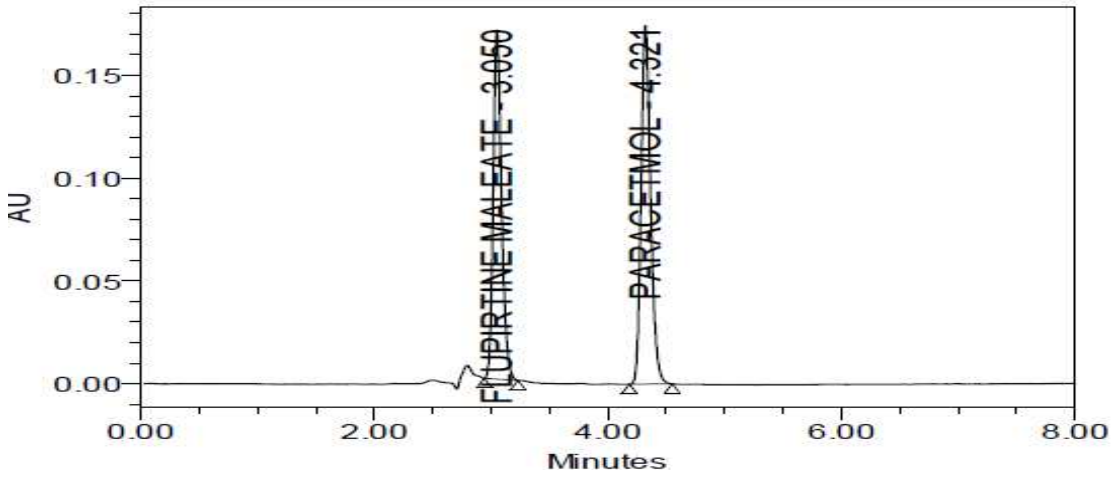


Fig no: 40 Chromatogram for increased flow rate

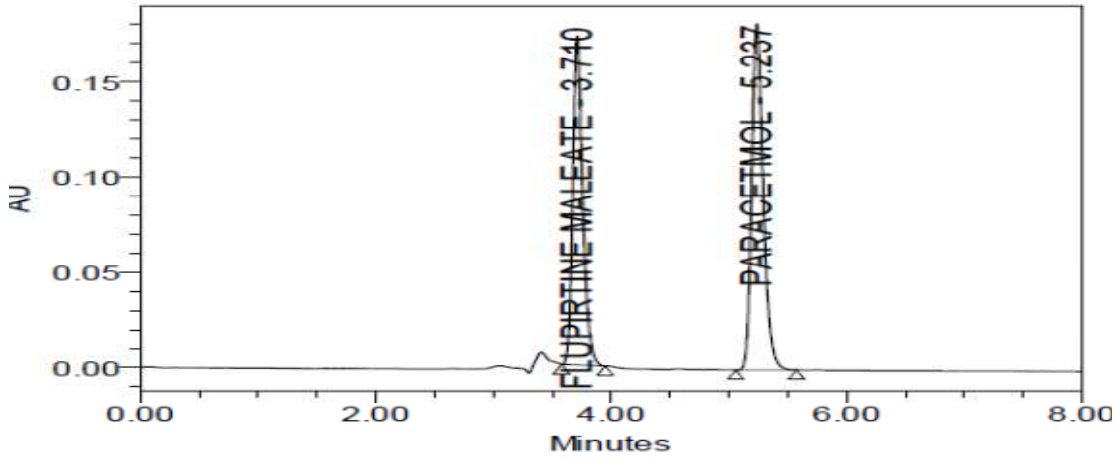


Fig no: 41 Chromatogram for decreased temperature

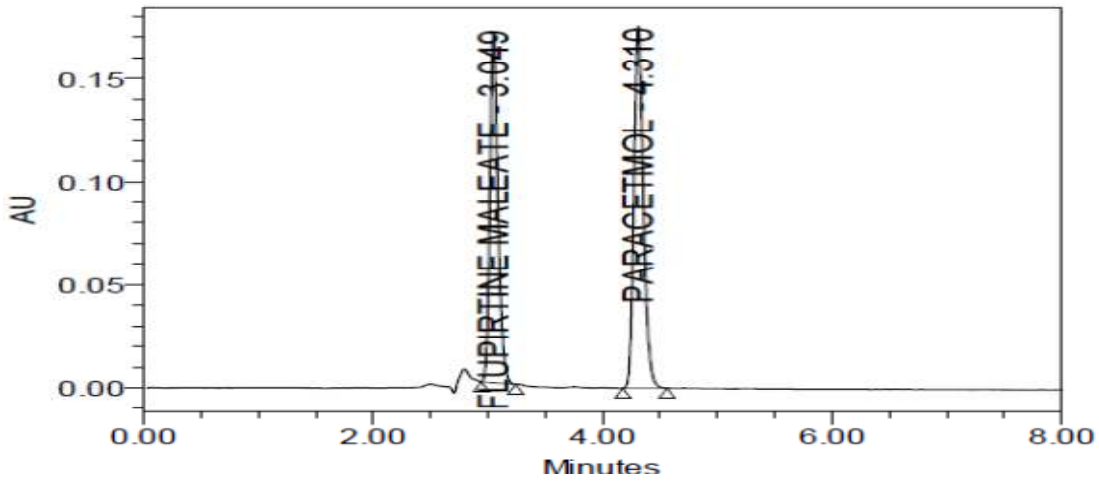


Fig no: 42 Chromatogram for increased temperature

RESULT

The results of Robustness of the present method had shown that changes made in the Flow and Temperature did not produce significant changes in analytical results which were presented in the above table. As the changes are not significant we can say that the method is Robust.

7. LIMIT OF DETCTION:

Minimum concentration of standard component in which the peak of the standard gets merged with noise called the LOD

$$LOD = 3.3* \sigma/S$$

Where;

σ = standard deviation

S = slope

LOD for Paracetmol = 3.769700

LOD for Flupirtine maleate = 3.182503

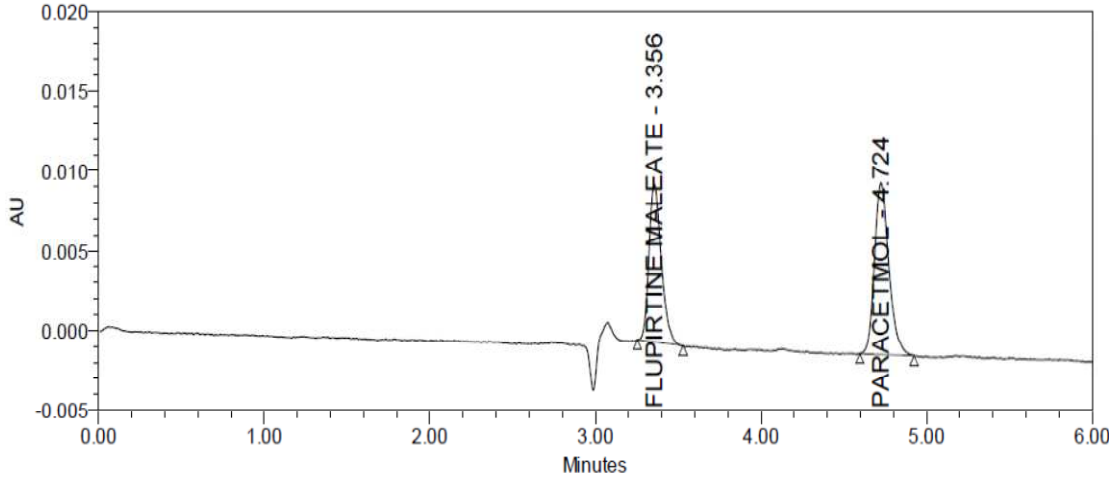


Fig no: 43 Chromatogram for LOD

8. LIMIT OF QUANTIFICATION:

Minimum concentration of standard component in which the peak of the standard gets detected and quantification

$$LOQ = 10*\sigma/S$$

Where;

σ = standard deviation

S = slope

LOQ for Paracetmol = 10.136540

LOQ for Flupirtine maleate = 10.259800

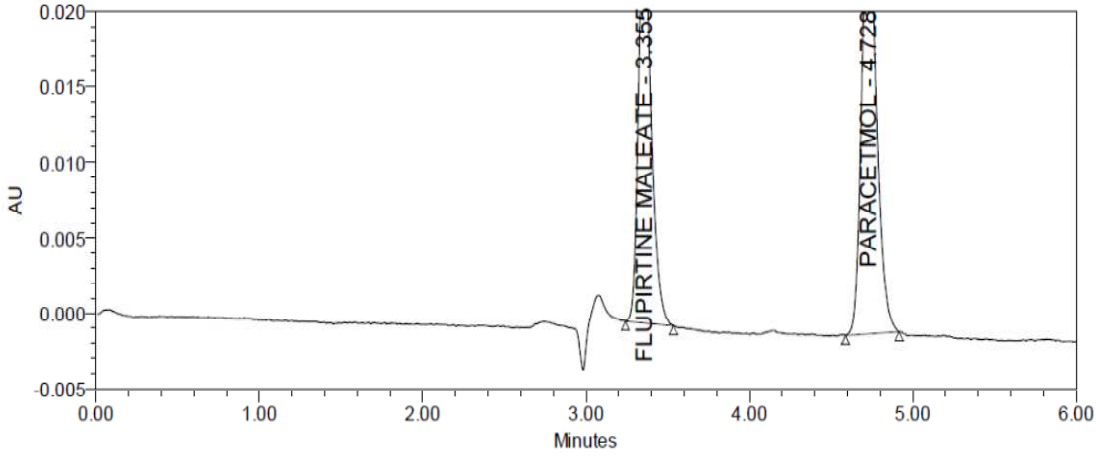


Fig no: 44 Chromatogram for LOQ

11. CONCLUSION

The study is focused to develop and validate HPLC methods for estimation of Paracetmol and Flupirtine male ate in tablet dosage form.

For routine analytical purpose it is desirable to establish methods capable of analyzing huge number of samples in a short time period with good robustness, accuracy and precision without any prior separation steps. HPLC method generates large amount of quality data, which serve as highly powerful and convenient analytical tool.

The method shows good reproducibility and good recovery. From the specificity studies, it was found that the developed methods were specific for Paracetmol and Flupirtine maleate. Al l the parameters of the methods.

12. SUMMARY OF PARACETMOL

Table no: 23 Summary of validation data for Paracetmol

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 13958 1.24 4.731 0.1	 Not less than 2500 Not more than2
2	Specificity a) Blank interference b) Placebo interference	Specific	Specific
3	Method precision(%RSD)	0.62	Not more than 2.0%
4	Linearity parameter Slope Correlation coefficient(r^2)	50-150 mcg/ml 43363 0.999	Not less than 0.999
5	Accuracy Mean % recovery	 101.00	 97 - 103%
6	Robustness a) Flow rate variation b) Temperature variation	All the system suitability parameters are within the limits.	

SUMMARY OF FLUPIRTINE MALEATE:

Table no: 24 Summary of validation data for Flupirtine maleate

S.NO	PARAMETER	RESULT	ACCEPTENCE CRITERIA
1	System suitability Theoretical plates Asymmetry Retention time %RSD	 9156 1.29 3.353 0.1	 Not less than 2500 Not more than 2
2	Specificity c) Blank interference d) Placebo interference	Specific	Specific
3	Method precision(%RSD)	0.19	Not more than 2.0%
4	Linearity parameter Slope Correlation coefficient(r^2)	50-150 mcg/ml 10569 0.999	Not less than 0.999
5	Accuracy Mean % recovery	 100.00	 97 - 103%
6	Robustness c) Flow rate variation d) Temperature variation	All the system suitability parameters are within the limits.	

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